Streptozotocin and sugar transport in pancreatic beta cell lines

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STREPTOZOTOCIN AND SUGAR TRANSPORT IN PANCREATIC BETA CELL LINES.

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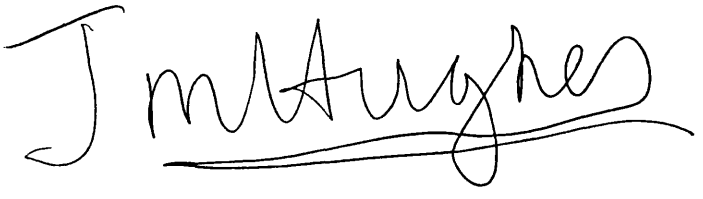

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Abbreviations

RIN	Rat insulinoma cell line
HIT	Syrian hamster induced cell line
HIT-T15	T-15 Subclone of the syrian hamster induced cell line
HITm2.2	2.2 subclone of the syrian hamster induced tumour cell line from mouse tissue
MIN	Mouse insulinoma cell line
MIN-6	Mouse insulinoma induced tumour cell line 6
MIN-7	Mouse insulinoma induced tumour cell line 7
Glut 1	Erythrocyte transporter
Glut 2	Liver or pancreatic transporter
Glut 3	Fetal muscle and brain transporter
Glut 4	Skeletal muscle and adipocyte transporter
Glut 5	intestinal cell transporter
RINr	Rat insulinoma cells from rat tissue
RINm	Rat insulinoma cells from mouse tissue
RINm5f	5f subclone of rat insulinoma cells
K_m	Michaelis Menten kinetic constant
DNA	Deoxyribose nucleic acid
cDNA	Complementary deoxyribose nucleic acid
mRNA	Message ribose nucleic acid
STZ	Streptozotocin
ALX	Alloxan

SV 40	Simian virus 40
T antigen	Tumour antigen
Hep G2	Human heptoma cell line
SDS PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
ASA-BMPA	4-azidosalicoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine
H1-H12	Hydrophobic alpha helical membrane spans 1-12
T_o	Outward facing sugar conformer
T_i	Inward facing sugar conformer
K_d	Dissociation constant
IDDM	Insulin dependant diabetes mellitus
NIDDM	Non-insulin dependant diabetes mellitus
MNU	N-nitrosomethylurea
NAD	Nicotinamide adenine dinucleotide
ADP	Adenosine diphosphate
V_{max}	Maximum velocity
K_s	Half saturation constant
HEPES	4-(2-Hydroxyethyl)-1-piperazineethane-sulphonic acid
PBS	Phosphate buffered saline
EDTA	Ethylene diaminetetraacetic acid
PMSF	Phenylmethoxysulphonalfluoride
DMSO	Dimethylsulphoxide
FCS	Heat inactivated fetal calf serum
NCS	Newborn calf serum

DMEM	Dulbeccos modified Eagles medium
RPMI-1640	Roswell Park Memorial Institute medium
K_i	Inhibition constant
GU	2-(3-methylureido)-2-deoxyglucopyranose
MIC	N-Methylisocyanate
Tlc	Thin layer chromatography
THF	Tetrahydrofuran

Summary

A characterization of the D-glucose transport systems in islet beta cell lines has been carried out. The cell lines used were HITm2.2, HIT-T15, RINm5f and MIN-6 and each of these lines were found to have different transport affinities and capacities to transport 6-deoxy-D-glucose and D-fructose. The ability to transport D-fructose was considered to be an important criteria of whether the cell line represented a model of the hexose transport properties of intact islets which are known to possess the glucose transporter isoform glut 2, which can transport D-fructose. All the cell lines rapidly transported 6-deoxy-D-glucose. Tracer concentrations (100 μM) were equilibrated with half times of under 30 seconds. The K_m and V_{max} for 6-deoxy-D-glucose were compared in each of the cell lines. The K_m and V_{max} values indicated the presence of a high affinity, high capacity transporter in the HIT and MIN-6 cell lines. Only the RINm5f cell line rapidly transported D-fructose and it is proposed that this cell line contains a separate transporter for D-fructose. From these studies it is inferred that the HITm2.2 and MIN-6 cell lines probably express the glut 2 isoform and that the RIN and HIT-T15 cell lines possess a mixture of transporter isoforms.

The sugar transport specificity requirements were investigated in the HITm2.2 and RINm5f cell lines. Several differences in specificity were noted which were interpreted in terms of interaction of the sugar analogues with glut 2 rather than the more common erythrocyte isoform, glut 1.

A radiolabelled analogue of the diabetogenic drug streptozotocin was synthesized. This compound is known to be cytotoxic for pancreatic islet beta cells and the hypothesis that this selective toxicity may be related to a selective uptake of the drug by the beta cells was explored using the beta cell lines HIT-T15 and MIN-6. In MIN-6 cells, the transport of streptozotocin was selectively inhibited by 6-deoxy-D-glucose and by D-fructose. 6-deoxy-D-glucose but not D-fructose inhibited transport into the HIT-T15 cell line. It is suggested that since the MIN-6 cells probably express glut 2, the streptozotocin uptake in these cells is probably via glut 2. The inability of D-fructose to inhibit the uptake in HIT-T15 cells is postulated to be due to the presence of an additional isoform for D-fructose in this cell line.

Although glut 2 may have a greater capacity to transport streptozotocin than the other isoforms it was discovered that streptozotocin also combined with glut 1. Studies were carried out in which cytochalasin B binding to glut 1 in erythrocyte membranes was determined in the

presence of streptozotocin. The streptozotocin was found to act as a competitive inhibitor of cytochalasin B binding.

The relationship between cytotoxicity and specificity for streptozotocin was examined in the HITm2.2 and RIN m5f cell lines The HITm2.2 cell line was found to be more sensitive to streptozotocin cytotoxicity than the RINm5f cell line. The cytotoxicity in the HITm2.2 cells was preventable by adding high concentrations of either D-glucose or 4,6-O-ethylidene-D-glucose to the incubation and growth media. It is postulated that the protective effect of 4,6-O-ethylidene-D-glucose is due to competition for entry of streptozotocin into the cells via the glucose transporter.

CONTENTS

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 1: INTRODUCTION.

1.1: The cellular composition of the pancreas.

The availability of *in vitro* islet cells isolated for experimental purposes have facilitated the study of beta cell metabolism. These islets are prepared from dissected pancreatic tissue which has been treated with collagenase. This procedure has enabled islet cells to be microdissected from other pancreatic tissue, with the aid of a microscope by careful microdissection (Lacy and Kostianovsky, 1967). Following microdissection these islet preparations can be maintained in cell culture for a limited time period. This method of islet extraction however, poses four major limitations. For experimental purposes it is both difficult and time consuming to prepare even small quantities of islets. The islets cellular and hormonal characteristics have been reported to change as a result of the extraction procedure. Results from experiments with *in vitro* islets have demonstrated a reduction in the amount of secreted insulin and finally, preparations of islet cell cultures are documented to be contaminated by the presence of non beta cells. As a result of these main disadvantages scientists have attempted to develop a permanent cell line which retains the primary functions of the beta cell.

The pancreas is a large gland situated behind the stomach, between the spleen and duodenum. This organ has an elongated structure divided into the head, body and tail regions. Its external secretion (exocrine) contains digestion enzymes, while its internal secretion (endocrine) is composed of insulin, glucagon and somatostatin. The endocrine function of the pancreas is performed by the islets of Langerhans, which comprise only 1 to 2 % of the whole organ. These islets of Langerhans are clusters of cells found scattered throughout the pancreas with approximately one million cells present in the adult human. In man the islets are round to ovoid structures and have been shown to display some variation in their size (20 to 300 μm in diameter). In addition islets have been observed to have an extensive contact with the vascular system of the pancreas.

There are several types of endocrine cell within each islet. These are the beta cells which produce insulin, alpha cells which produce glucagon and the delta cells which produce somatostatin. The proportions of each type of cell are as follows. Each individual islet is normally comprised of 60 to 70 % beta cells and 5 to 10 % delta cells.

In the last decade several pancreatic beta cell lines have been established from a variety of animal tissue sources. I have examined whether these cell lines can be

used as a model in which to study the function and morphology of the beta cell with respect to the transport of specific D-glucose analogues.

Organisms regulate their internal cellular environment by the exchange of physiological and chemical information with their external environment. This cellular regulation is achieved by a combination of co-ordinated intramembrane channels and carrier protein complexes. The D-glucose molecule provides a major source of metabolic energy. Therefore, a facilitative glucose transport system is present in all procaryotic and eucaryotic cells. In complex eucaryotic organisms with tissues which perform specific functions, there exists a family of glucose transporter proteins. These transporters have a primary sequence homology with one another and a tissue-specific pattern of expression. It has become apparent that this unique protein family, whose primary function is to transport D-glucose, has been modified to regulate the metabolism of D-glucose in each specific tissue.

1.2: Development and characteristics of cultured pancreatic beta cells

1.2.1: The rat insulinoma cell line (RIN)

The RIN cell line was established by two collaborative scientific groups (Gazdar et al., 1980; Lambert et al., 1972). Gazdar's group (Gazdar et al., 1980) were responsible for the establishment of the RIN cell line which originated from neoplastic rat islet tissue (Chick et al., 1977). In these cells, tumours were induced by subjecting inbred rats (New England Deaconess Hospital) to X-ray radiation. This irradiation produced transformed cells which were maintained by serial transplantation. After nine transplants of the neoplastic tissue, the tumour was finally heterotransplanted into an athymic nude mouse. The cell lines were derived from either the rat or nude mouse transplants. They are called rat insulinoma cells derived from rat (RINr) and rat insulinoma cells derived from mouse (RINm).

RIN m5f cells have an epitheloid structure. The cells have been observed to double their population after a time period of 60 to 80 hours (Gazdar et al., 1980). The quantity of insulin secreted by the RIN m5f clone was found to be comparable to that of the parent RINm cell. Both of these cell types were recorded to have secreted

insulin at 8ng/10^6 cells/24 hours (Gazdar et al., 1980). The secretion of insulin in response to a variety of secretagogues which include calcium, tolbutamide, theophylline and glucagon has been documented by various research groups (Bhathena et al., 1984). Praz et al., (1983) have demonstrated that the insulin content of RINm clones at higher passage numbers was equivalent to only 1% of the total insulin measured in native rat beta cells.

Malaisse et al., (1986) studied the uptake of the non-metabolised D-glucose analogue 3-0-methyl-D-glucose into the RIN m5f cell line. These transport experiments were performed at a range of different time periods and temperatures. This group proposed that 3-0-methyl-D-glucose was not fully equilibrated at any of the temperatures or time periods used in the study. The transport of D-glucose was shown to be inhibited by 3-0-methyl-D-glucose. From these results obtained from their particular RINm cell line, Malaisse et al., (1986) postulated that there may be more than one sugar transporter protein in this particular cell line. This group also showed that cytochalasin B and phlorrizin were both potent inhibitors of transport. The observation of a loss of the ability of RIN cells to fully equilibrate D-glucose analogues across the cell membrane was contested by other groups. Trautman and Wollheim, (1987) demonstrated an efficient hexose transporter system

displaying both a high capacity and low affinity for 3-O-methyl-D-glucose in RIN m5f cells. In this work the cells were equilibrated with 3-O-methyl-D-glucose and the Michaelis Menten constant (K_m) measured was found to be 32 mM. This contrasts with an earlier finding of Malaisse et al., (1986) who reported a K_m value of 2mM.

Thorens et al., (1988) found that a subclone of the RINm demonstrated a poor insulin secretory response to glucose. These investigators attributed this to the expression in these cells of the Glut 1 transporter. Recently Shibasaki et al., (1990) have developed a stable RINr cell line that over-expresses a complementary deoxyribose nucleic acid (cDNA) which codes for the erythrocyte type transporter (Glut 1). This cell line was observed to exhibit both an enhanced and constitutive secretion of insulin.

Certain RINr cell lines were reported to be resistant to the toxic effect of the diabetogenic compounds streptozotocin (STZ) and alloxan (ALX) (Ledoux and Wilson, 1984; Sener and Malaisse, 1985). These investigators proposed that both STZ and ALX were taken up by the cell. Part of the resistance to the diabetogenic effects of both of these compounds has been attributed to their reduced transport into specific RIN cells (Sener and Malaisse, 1985). It was unclear from these studies whether this uptake was specifically via the glucose transporter.

1.2.2: The Syrian hamster induced tumour cell line (HIT)

This cell line was established by Santerre et al., (1981). These authors isolated islet cells from the Syrian hamster and treated them with ethylmethanesulphonate. Treated islets were then transformed and subsequently immortalised with simian virus-40 (SV40). Hamster induced tumour cells (HIT) were found to secrete insulin at a rate of 90 ng/ 10^6 cells. The quantity of insulin released by HIT cells has been shown to vary with both passage and growth. This insulin release has been demonstrated to decline over an 11 month period up to passage 88 (Santerre et al., 1981; Zhang et al., 1989). One of the parent subclones is the HIT-T15 m 2.2.2 often abbreviated to HIT m2.2 (Edlund et al., 1985). In recent years both the parent HIT-T15 and the HITm2.2 subclone have been used as models in which to study the beta cell. These cell lines have also been demonstrated to secrete insulin in response to amino acids and a variety of other secretagogues (Hill and Boyd, 1985).

Some of the transport properties of the sugar transporter in HIT cells have been described by Meglasson (et al., 1986). This group demonstrated that 3-O-methyl-D-glucose was equilibrated within 20 minutes. This was considered a slow rate of transport when

compared with some other insulinoma cell lines. In these insulinoma cells 3-0-methyl-D-glucose equilibration was completed in 30 seconds. Ashcroft and Stubbs, (1987) confirmed that sugar transport into the HIT-TI5 cell line proceeded at a slow rate.

The classical sugar transport inhibitors phloretin and phlorrizin as well as the fungal inhibitor cytochalasin B, inhibited the utilisation of D-glucose within the HIT cell (Ashcroft et al., 1980). Ashcroft and Stubbs, (1987) also reported that neither L-glucose nor N-acetyl-D-glucosamine were transported by the HIT cell. D-galactose, 6-deoxy-D-galactose, goldthio-D-glucose and D-fructose sugars only competed weakly with D-glucose for entry into the cell. In these cells, insulin release in response to glucose was inhibited by both phloretin and cytochalasin B (Ashcroft and Stubbs, 1987). It has been observed more recently in HIT cells exposed to high glucose concentrations (Purrello et al., 1991), that both of the Glut 1 and liver/ pancreatic type transporter (Glut 2) mRNA levels are reduced. This mRNA reduction was associated with an impaired glucose induced insulin secretory mechanism. From this study Purrello (et al.,1991), inferred that the HIT cell glucose transporter isoforms were regulated by the glucose molecule itself. Purrello proposed that a mechanism may exist whereby D-glucose down regulated its own transport.

1.2.3: The mouse insulinoma cell line (MIN)

A recent transgenic method (Sarvetnick et al., 1988) has provided an experimental system which targets the expression of a specific oncogene in the transgenic mouse. As a direct result of this gene transformation, immortalised cell lines with differentiated phenotypes have been established.

The pancreatic beta cell lines which can be obtained by this strategy have retained many of the characteristics of the original beta cell. Two groups have reported upon the establishment of cell lines which have been derived from the SV40 T antigen transgenic mouse. One of these cell lines was the beta Tumour Cell 1 (Efrat et al., 1988). These cells exhibited a high level of insulin secretion, while at the same time the cells demonstrated a high sensitivity to glucose stimulation. In comparison another beta cell line derived from a SV40 T antigen transgenic mouse (Gilligan et al., 1989), showed no response to any glucose stimulation.

Two specific cell lines have been established from insulinomas. These lines were obtained by the specific targeted expression of the SV 40 T antigen gene in transgenic mice. Two such tumour induced mice (IT 6 and IT 7) developed pancreatic insulinomas at 13 weeks of age and the resulting murine cell lines were assigned the names MIN-6 and MIN-7. These lines were

shown to produce significant quantities of both insulin and T antigen while they also retained the morphological characteristics of the pancreatic beta cell (Miyazaki et al., 1990). Of the two murine cell lines, the MIN-6 cells secreted insulin in response to glucose at a rate which was comparable to mice islets maintained in culture. However MIN-7 cells showed a negligible insulin secretion capacity. Miyazaki et al., (1990) have reported that both of these cell lines contained mRNA which coded for the Glut 2 transporter. The MIN-6 cell line was shown to possess higher Glut 2 mRNA levels than MIN-7. The latter cell line was reported to contain large quantities of Glut 1 mRNA.

1.3: The erythrocyte transporter (Glut 1)

During the last decade, the advent of molecular biological techniques have enabled the family of membrane glucose transport proteins to be cloned. There are now known to be three main categories of sugar transporter. Characterisation into these groups is based on certain key observed functional differences. These categories are the sodium dependent, sodium independent and the insulin sensitive type of transporter. The erythrocyte, liver and beta cell transporters belong to the sodium independent category of this protein family.

Table 1.1: The five types of human glucose transporter (Gould and Bell, 1990).

Designation (common name)	Size (no. of amino acids)	Major sites of expression	Chromosomal location
(a) Facilitative glucose transporters			
(1) GLUT 1 (Erythrocyte, HepG2, brain)	492	Fetal tissues, brain, kidney and colon	1
(2) GLUT 2 (Liver)	524	Liver, β-cell, kidney and small intestine	3
(3) GLUT 3 (Fetal muscle)	496	Many tissues including brain, placenta and kidney	12
(4) GLUT 4 (Muscle/adipocyte, insulin-regulatable)	509	Skeletal muscle, heart and adipocytes	17
(5) GLUT 5 (Small intestine)	501	Small intestine	1
(b) Na^+/glucose co-transporter or symporter			
(1) SGLT1 (Na^+/glucose co-transporter)	664	Small intestine	22

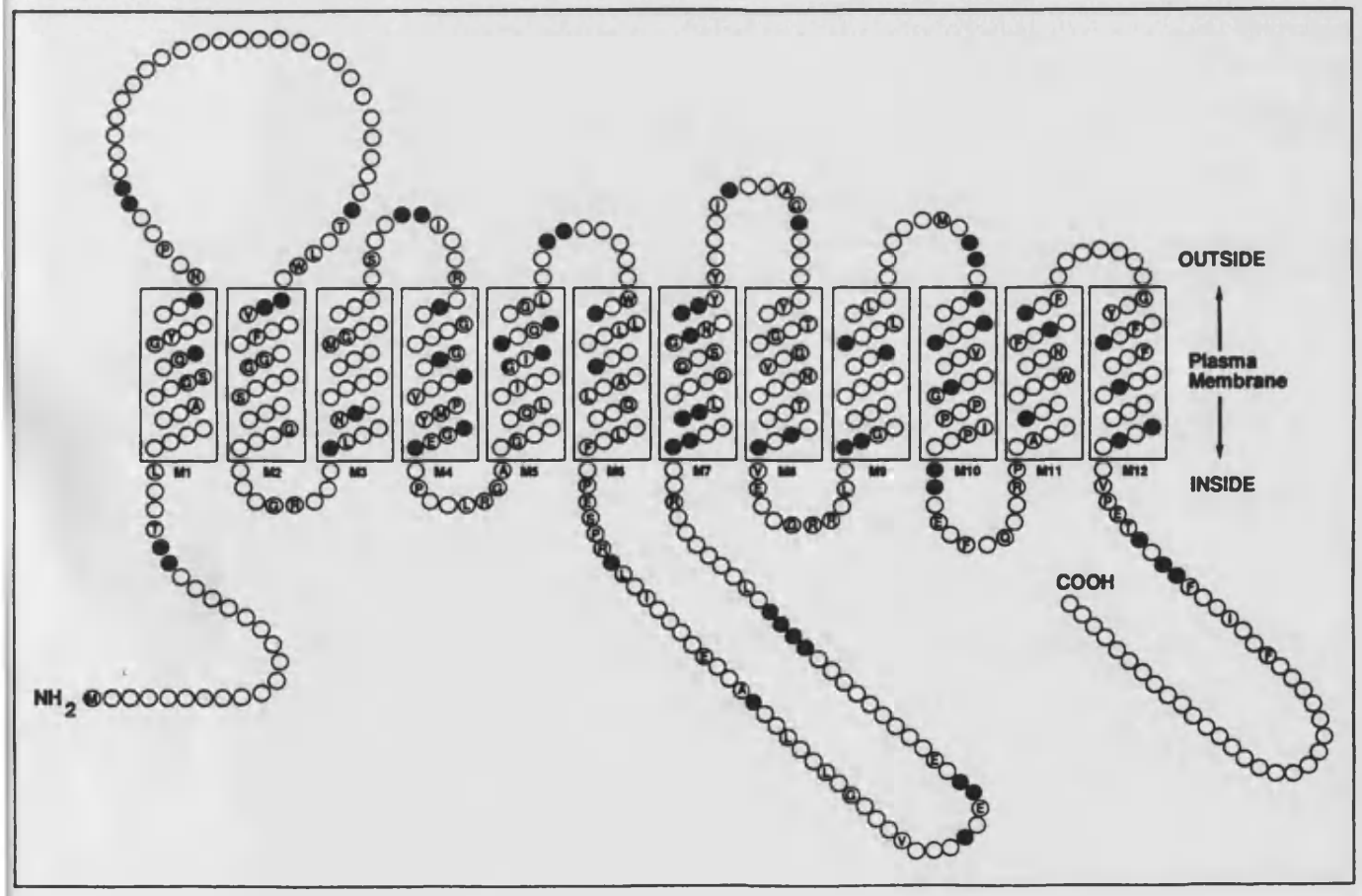
It is now apparent that there are a family of genes which are located on different chromosomes. These genes code for a variety of sugar transport proteins which are expressed in the types of cell shown in Table 1.1. To date at least five different transporter isoforms have been isolated and their respective amino acid sequences have been deduced from their cloned cDNA's. These protein isoforms are designated Glut 1 to 5.

Glut 1 has been the most extensively studied of all the transporters. The Glut 1 transporter is proposed to be an integral membrane protein of between 45 to 55 kDa. Mueckler et al., (1985) screened the gene library with a specific antibody raised against a partially purified Glut 1 protein. The Glut 1 sequence coded for a 492 amino acid protein which was similar but not identical to a partial sequence from an earlier purified preparation reported by Baldwin et al., (1982). HepG2 cells, erythrocytes and rat brain were shown to contain the Glut 1 protein (Birubaum et al., 1986). The brain Glut 1 sequence was 98 % identical to the Glut 1 sequence obtained from the HepG2 cell line.

The mRNA coding for the Glut 1 transporter has been subsequently identified in most tissues including fetal tissue and the placenta. In adults, Glut 1 mRNA was found in the brain, kidney, colon and most cultured cell lines. Levels of the Glut 1 mRNA in the liver and skeletal

Figure 1.1: A model showing the proposed orientation of the facilitative glucose transporter within the plasma membrane.

There are twelve transmembrane helices which are shown as outlined boxes, numbered M1 to M12. There is a potential attachment site for a N-linked oligosaccharide in the extracellular loop connecting helices M1 and M2. This oligosaccharide is present in each of the five mammalian facilitative glucose transporter isoforms. Identical amino acids in the human Glut 1 to Glut 5 are indicated using their single letter abbreviation (D, E; F, Y, W; I, L, V, M; K, R; N, Q; and S, T). Chemically similar residues are denoted by the black circles. The N- and C-terminal domains as well as the exofacial loop between M1 and M2, differ in both sequence and size between the transporter isoforms (Gould and Bell, 1990).



muscle tissues were however found to be low (Fukumoto et al., 1988a).

Mueckler et al., (1985) proposed a two dimensional model for the orientation of the sugar transporter within the membrane. This was based on a sequential arrangement of the amino acids according to their hydrophobicity as outlined in Figure 1.1. The structure of the sugar transporter is reputedly organised into three main domains which are outlined below:

Domain 1 consists of twelve alpha helices each spanning the lipid bilayer. Both the amino and carboxyl end terminal amino acids are proposed to be located within the cytoplasm. Domain 2 comprises 65 highly charged hydrophilic amino acids situated endofacially. Domain 3 is a 33 amino acid portion situated exofacially. Domain 3 contains the asparagine amino acid which is linked to an oligosaccharride when the protein is in its native state.

Studies using peptide specific antibodies carried out by Davies et al., (1987) have confirmed a number of salient features of the transporter model which are outlined in figure 1.1. Further information about the characteristics of the transporter have been confirmed by epitope mapping studies using proteolytic digestion and specific antibodies (Cairns et al., 1984). Trypsin was used to cleave the cytoplasmic face of Glut 1. This treatment resulted in the production of two membrane

fragments. One of these cleaved fragments contained the asparagine-linked oligosaccharride while the other fragment contained the cytochalasin B binding site. Spectroscopic studies performed by Alvarez et al., (1987) have also confirmed that Glut 1 possess mainly alpha helices together with some random coil and beta sheet structure. In addition, the extracellular protein domains have been shown to contain some alpha helical structure (Cairns et al., 1987).

1.3.1: Reconstitution studies and the covalent labelling of the Glut 1 Transporter

Studies have been performed in which erythrocyte Glut 1 has been purified and reconstituted by using surfactants and chromatographic techniques (Kasahara and Hinkle, 1977). In 1981 Wheeler and Hinkle (1981), used purified erythrocyte Glut 1 and showed that sugar uptake was inhibited by cytochalasin B, mercury chloride and phloretin. In addition the purified transporter from erythrocytes has been shown to bind 0.5 moles of cytochalasin B per mole of protein (Sogin and Hinkle, 1978). Baldwin et al., (1982) purified Glut 1 from erythrocytes using octylglucoside and found that 0.7 moles of cytochalasin B bound per mole of transporter, indicating a possible 1:1 stoichiometry. Further work using reconstitution studies with liposomes

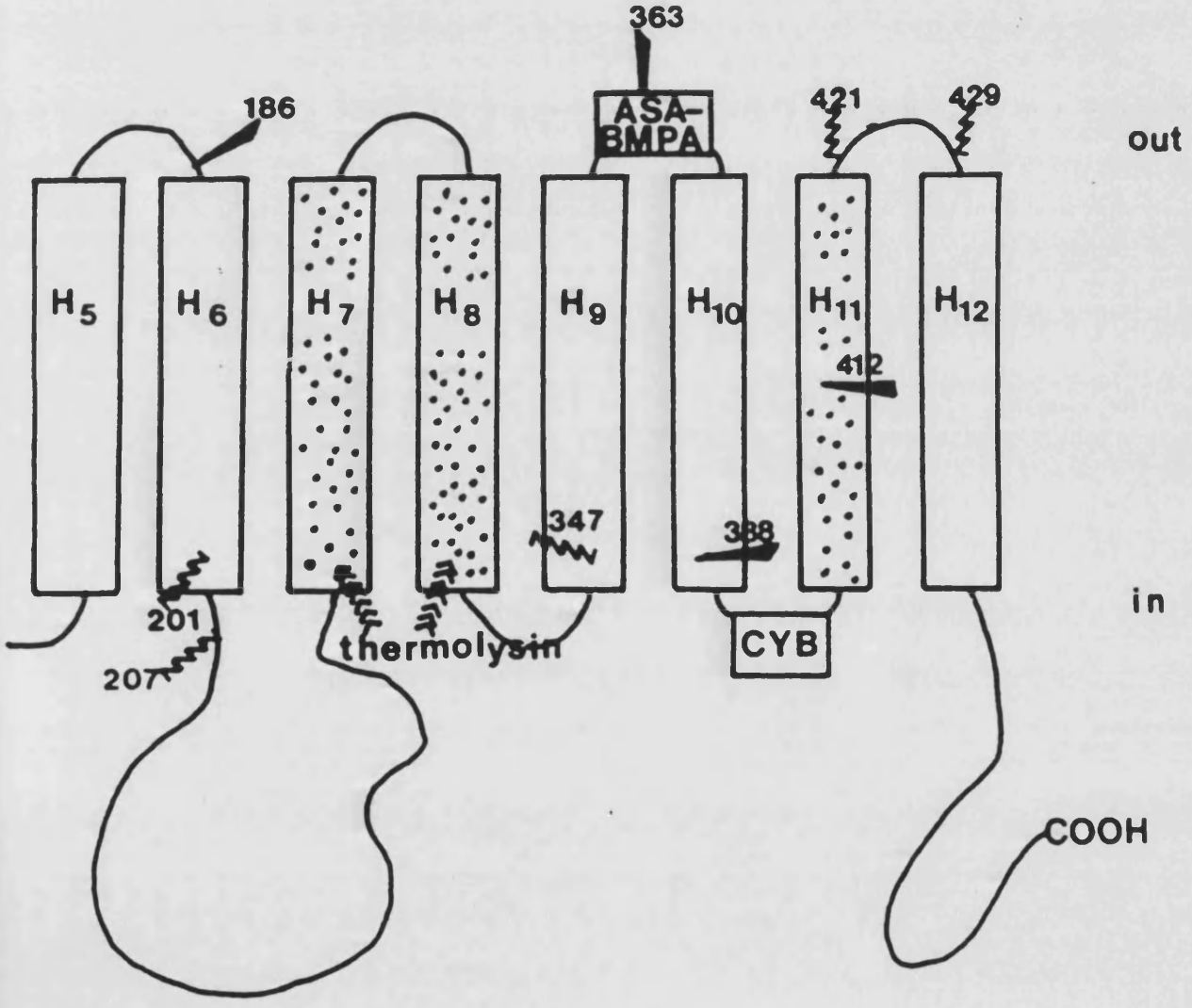
has shown a functional protein which mediates D-glucose uptake, with kinetics that are proposed to be indistinguishable from the transporter in intact cells (Carruthers and Melchoir, 1984).

Deziel et al., (1984) has shown how covalent labels can be used to characterise Glut 1. These labels include glycosylisothiocyanate, 1-fluoro-2,4-dinitrobenzene and cytochalasin B. The latter can be used as a specific photolabel. Following irradiation with ultraviolet light cytochalasin B can be used to label the transporter endofacially with an 8% efficiency (Deziel et al., 1984).

Exofacial labelling reagents have been developed with a photoreactive group at position 4 on the sugar ring. Bis-hexoses were synthesized. These labelled the exofacial binding site of the transporter. One key specific exofacial label, the azidosalicoyl derivative of bis-D-mannos-4-yloxy-2-propylamine (ASA-BMPA) has been shown to label the Glut 1 transporter peptide (Holman et al., 1986).

Cytochalasin B photolabelling followed by proteolytic fragmentation has been used to produce a map of the inside and outside binding sites as illustrated in figure 1.2 (Holman and Rees, 1987). The cytochalasin B binding site has been specifically assigned to the inside of the hydrophobic alpha helical membrane span 10 (H-10), with tryptophan 412 reputed to be the labelled residue (Cairns et al., 1987). The sugar binding core of the

Figure 1.2: The proposed location of the exofacial ASA-BMPA and endofacial cytochalasin B-binding sites on the erythrocyte hexose transporter (Holman, 1989).



transporter is proposed to possess both an internal and an external (ASA-BMPA labelled portion) binding site. A transmembrane glucose channel is proposed to be located between H-7 and H-10.

1.3.2: The hydrogen bonding specificity of the Glut 1 transporter

Studies investigating the hydrogen bonding specificity between glucose and the transporter have been performed by Lefevre (Lefevre and Marshall, 1958). These investigators have proposed that the transporter preferred the chair conformation of the sugar. Kahlenberg and Dolansky, (1972) inferred that several hydroxyl groups upon the sugar ring were involved in binding. This was confirmed by Barnett et al., 1973. This group studied analogues in which hydroxyl groups were replaced with hydrogen and fluorine at carbons 1, 2, 3, 4 and 6. These authors demonstrated that all of the sugar analogue derivatives bound to the protein in the beta pyranose form. Hydroxyl groups on the 1, 3 and 6 carbons were also proposed to be required for binding to the transporter (Barnett et al., 1973). Barnett et al., (1975) inserted bulky hydrophobic substituents onto D-glucose at the 1, 4 and 6 positions and proposed the existence of a protein pocket. It has been shown that when glucose approaches the transporter exofacially or endofacially, carbon 1 and

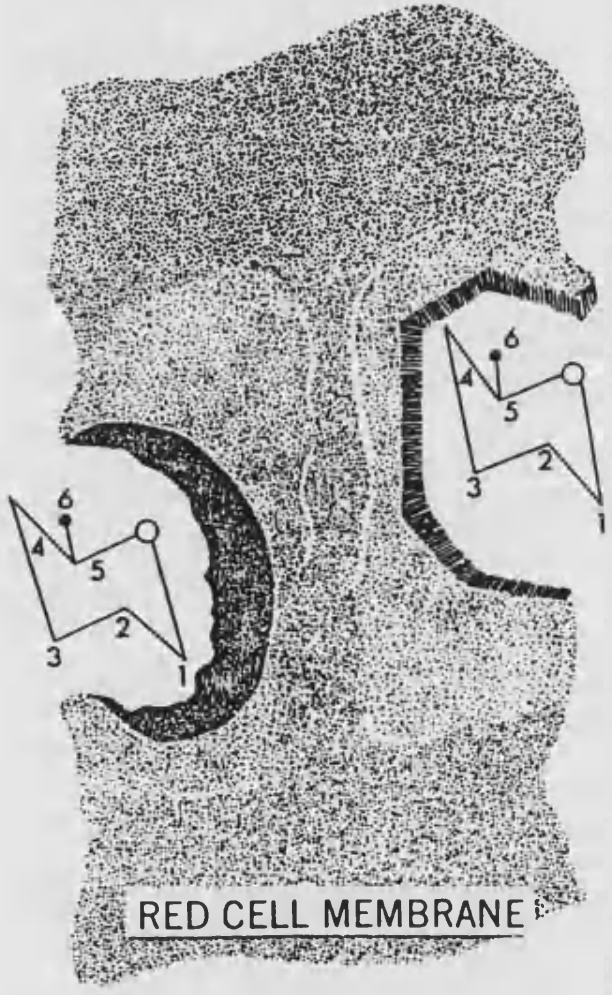
6 interacts first with the inward or outward binding conformer respectively as in figure 1.3. The hydrogen bonds at the binding site may be provided by helices 7, 8 and 11. The hydrophobic cleft may be composed of helices 9 and 10 (Silverman, 1991).

Data from hydrogen exchange experiments have been used to predict that ligand binding would collapse a water filled channel (Harvey et al., 1976). Alkyl groups substituted for the hydroxyls upon the sugar ring were proposed to reduce the binding of glucose by steric interference.

1.3.3: The mechanism and kinetics of glucose binding to the Glut 1 transporter site

D-glucose is transported by a passive diffusion process. The K_m for D-glucose uptake has been shown to be 1.6 mM (Block, 1974; Appleman and Leinhard, 1985; Lin and Spudich, 1974). Lowe and Walmsley, (1986) proposed that the transporter adopts both mutually exclusive inward and outward facing conformations. This idea has been supported by Holman and Rees, (1987) who used thermolysin cleavage to promote cytochalasin B binding and exclude the ASA-BMPA label from binding. In this study cytochalasin B was shown to bind to the inward-facing cleavage-susceptible conformer. Cytochalasin B competitively inhibits D-glucose efflux and therefore

Figure 1.3: A diagrammatic representation of the proposed binding of the pyranose substrate to the hydrophobic cleft of the erythrocyte glucose transporter (Silverman, 1991).



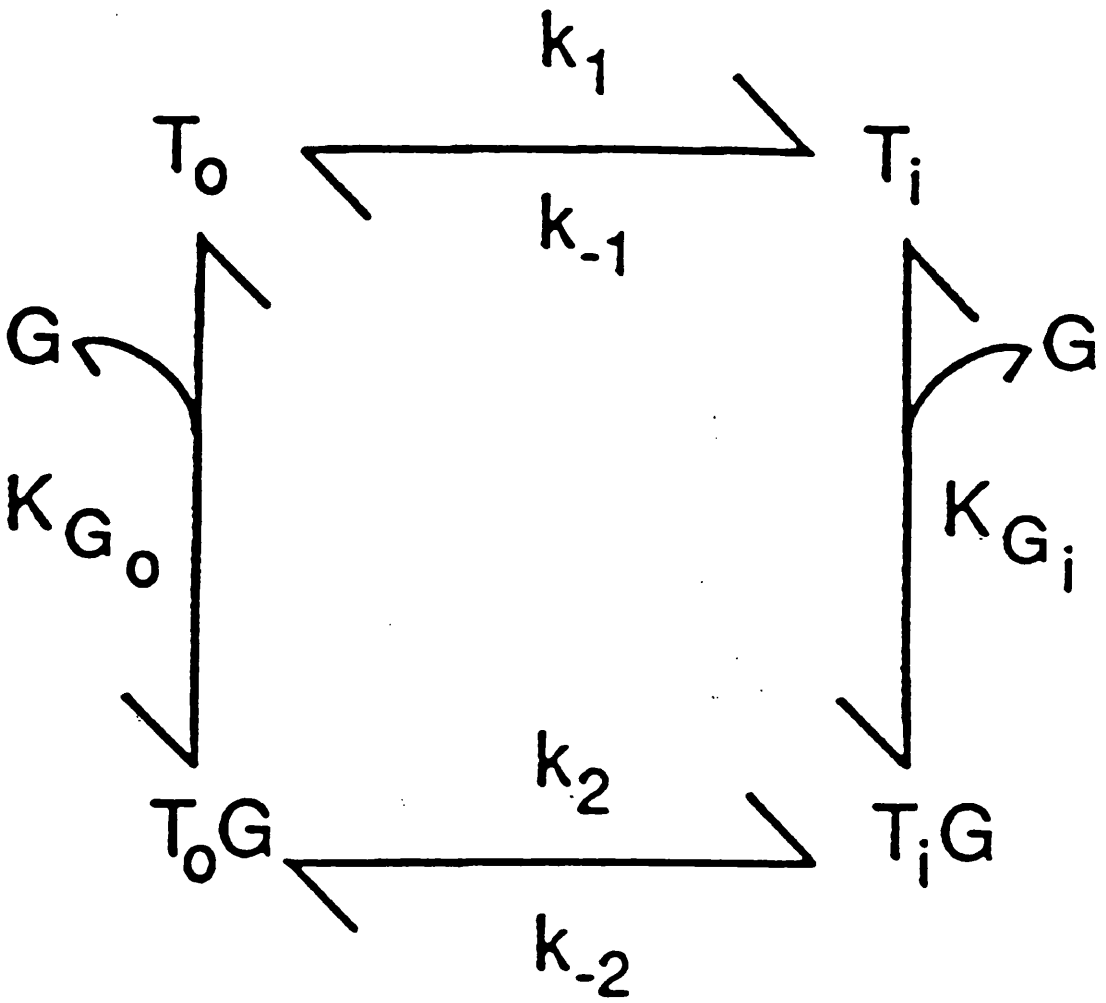
binds to the inward-facing conformer. Cytochalasin B was shown to inhibit the influx of D-glucose.

Further evidence which supports the alternate conformer model was provided by ligand binding studies showing that the inward and outward conformers were mutually exclusive. In this work 4,6-O-ethylidene-D-glucose was shown to bind exofacially and to prevent cytochalasin B binding (Deves and Krupka, 1978; Gorga and Leinhard, 1981).

Spectroscopic studies have revealed that D-glucose, cytochalasin B and 4,6-O-ethylidene-D-glucose all attenuate fluorescent emissions (Pawagi and Deber, 1990; Gorga and Leinhard, 1985). This has been attributed to a conformational change due to a tryptophan transition from a hydrophilic to a hydrophobic environment. This change possibly involves tryptophan 363, 388 or 412. Tryptophan 388 was proposed to be the candidate due to its proximity to the dynamic transport segment shown in figure 1.2. A transient fluorescence due to 4,6-O-ethylidene-D-glucose binding was attributed to a transporter reorientation from the inward to the outward facing conformer (Appleman and Leinhard, 1985).

Evidence gathered from pre-steadystate stopped-flow kinetic experiments (Appleman and Leinhard, 1985) have supported an alternating conformational model for D-glucose transport (figure 1.4).

Figure 1.4: A schematic representation of the kinetic model of the glucose transporter. The k's represent rate constants for the substrate and protein (receptor) dissociation. G and T refer to the glucose and transporter concentrations. T_o represents the outward facing transporter conformation and T_i the inward facing conformer (Silverman, 1991).



In the alternating conformation model for transport the protein is believed to oscillate between two specific conformations (Lowe and Walmsley, 1986). These are both an extracellular and an intracellular conformer state. Upon occupation of the binding site, a conformational change will occur resulting in the translocation of the bound glucose across the membrane.

A second model proposes that the two substrate binding sites are simultaneously present (Carruthers, 1986). This model is a more complex kinetic analysis. If however the carriers exist as oligomeric proteins in their native state within the membrane then complicated kinetic behaviour may take place.

1.4: The liver type glucose transporter (Glut 2)

The liver plays a crucial role in maintaining blood glucose homoeostasis. After feeding it absorbs glucose for the synthesis and storage of glycogen. The liver can synthesize glucose from amino acids. In the liver insulin increases glucose utilisation but not its uptake. This contrasts with fat and muscle tissue where uptake is stimulated by insulin (Calderhead and Leinhard, 1988).

Biochemical and molecular biological studies has shown a distinct type of liver transporter (Glut 2) with K_ms for D-glucose and cytochalasin B ten fold higher than Glut 1 (Axelrod and Pilch, 1983). Glut 2 has been shown

to be present in the beta cell (Fukumoto et al., 1988b; Thorens et al., 1988; Permutt et al., 1989).

Glut 2 is unique as it releases glucose during fasting. It also has a high K_m for D-glucose (Elliot and Craik, 1982). This high K_m of glut 2 ensures that the transporter operates in the linear portion of the substrate velocity curve and that the glucose flux will change linearly according to the blood glucose level. cDNA clones have been isolated which code for both the human and rat Glut 2 proteins. The Glut 2 sequence possesses a 55.5 % identity to Glut 1, with an 82 % identity between the human and rat transporters. Analysis of the predicted primary structure has indicated that Glut 2 possesses the same topological organisation as Glut 1. Expression of Glut 2 in an *Escherichia coli* (E. coli) defective glucose transport strain has shown that the protein is a transporter with the expected stereospecificity (Thorens et al., 1988).

1.5: The pancreatic islet beta cell or Glut 2 transporter

Hellman (et al., 1971) provided the initial evidence for the mediated transport of D-glucose into the beta cell. Sugar uptake into freely suspended islets was shown to reach equilibrium within 1 minute (Lernmark et al., 1975; Lernmark et al., 1976). Gorus et al., (1984) have shown that 3-O-methyl-D-glucose is transported at a rate of

402 pmol of sugar per minute per cell. Beta cells have been shown to equilibrate 3-0-methyl-D-glucose within 2 minutes in contrast to the alpha cells where full equilibration did not occur.

In 1989 Orci et al., (1989) performed a study of the Glut 2 topography and observed that the transporter was confined to specific domains within the plasma membrane of islets. This group, using data from labelled electron microscopy techniques, have indicated that the transporter is responsible for the flow of D-glucose within the islet. It was proposed that Glut 2 appears to direct glucose to regions distal from the capillaries. 3-0-methyl-D-glucose has been shown to be 90% equilibrated across the dispersed islet cell within an incubation time of 1 minute (Johnson et al., 1990 b). Kinetic analysis has revealed that glucose transport in islets occurs via two kinetically distinct components. The first component has a K_m of 17mM which was attributed as being associated with the presence of Glut 2. The other component has a K_m of 1.4 mM and is probably associated with the presence of Glut 1. In this work it was also demonstrated that distinct cytochalasin B binding constants existed which were attributable to Glut 2 and Glut 1. This data suggested that Glut 2 was present in higher amounts than Glut 1.

Chen (et al., 1990) investigated whether the Glut 2 protein was constitutively expressed or regulated in the

beta cell. In animal studies, these authors have recorded that after the induction of hypoglycaemia, the K_m for 3-0-methyl-D-glucose is reduced. There is an associated reduction in the Glut 2 mRNA level. It was inferred the expression of Glut 2 could be regulated by the circulating blood glucose level. Yasuda (et al., 1992) also confirmed this. Under hyperglycaemic conditions the Glut 2 message was shown to increase. In cultured islets, Glut 2 mRNA levels were shown to increase following incubation at high D-glucose, D-mannose and D-fructose concentrations (Asano et al., 1992). In this study however, 3-0-methyl-D-glucose, 2-deoxy-D-glucose and D-sucrose had no effect.

A detailed kinetic investigation of the Glut 2 transporter was performed by Gould (et al., 1991). His laboratory have successfully expressed Glut 2 in the Xenopus oocyte expression system. They measured 3-0-methyl-D-glucose and 2-deoxy-D-glucose transport. 2-deoxy-D-glucose uptake was found to be inhibited by D-glucose but not L-glucose and D-galactose. D-mannose and D-fructose were also demonstrated to be transported by human Glut 2. Gould also obtained K_m values for Glut 2 with respect to transported 3-0-methyl-D-glucose (42 mM), 2-deoxy-D-glucose (13 mM) and D-glucose (66 mM) respectively. These kinetic values were shown to be different to those of Glut 1. Sener (et al., 1984) have also shown that the beta cell transports D-fructose.

A number of cell lines have also been reported to contain the Glut 2 transporter. A study of transporter gene expression in a Syrian Hamster pancreatic beta cell line has revealed the existence of both Glut 1 and Glut 2 mRNA (Purrello, 1991). When this cell line was maintained in tissue culture at a high glucose concentration the mRNA levels for Glut 1 and Glut 2 were found to be reduced. This effect was correlated with a reduction in the cells insulin secretion capability. Miyazaki (et al., 1990) generated a cell line (MIN-6) that retained the ability to secrete insulin in response to a glucose challenge. Here Glut 2 was shown to be the major transport protein. Evidence for this was based on the presence of Glut 2 mRNA in these MIN-6 cells.

1.6: Glut 2 transporter dysfunction and a possible link with diabetes

In the Western Hemisphere diabetes is estimated to affect 2% of the total population. From this populace total, 10 to 25% consists of a clinically defined specific autoimmune disorder which destroys the insulin secreting beta cells. This disorder is called Type 1 or insulin dependant diabetes mellitus (IDDM). As the "insulin dependent" name infers patients suffering from this condition require insulin replacement therapy. Such therapy will thus prevent the advanced diabetic symptoms

of ketoacidosis, coma and death. In the remaining 75 to 90% the diabetes is somewhat attenuated and hyperglycaemia is caused by a failure of beta cells to meet increased demands for insulin (Type 2 or Non-insulin Dependent Diabetes). The reason why these cells cannot meet this demand for insulin is as yet undefined. Table 1.2 outlines the primary symptoms associated with these two disease states.

Some evidence linking Diabetes directly to a defect in the transport of glucose has originated from antibody/ islet transport experiments. In these studies it was found that within 24 hours of the onset of autoimmune diabetes, D-glucose transport is reduced by 90% (Tominga et al., 1986). This loss in transport activity has been shown to be accompanied by the reduction of glucose stimulated insulin secretion. In this study however, a specific response to arginine is still retained. From this and other such observations it has been proposed that a defect in the transport function, irrespective of the cause, would impair the beta cells ability to respond to hyperglycaemia.

Chen (et al., 1990) reported that the Glut 2 protein is down regulated by chronic hyperinsulinemia. In this work, at concentrations of glucose above normal fasting levels, glucose transport was impaired by the absence of Glut 2. This is further evidence that an absence or impairment of Glut 2 would result in beta cells being

Table 1.2: A comparison between the two clinically defined forms of diabetes mellitus. These are type 1 insulin dependent diabetes mellitus (IDDM) and type 2 non-insulin dependent diabetes mellitus (NIDDM) (Unger, 1991).

Characteristic	IDDM	NIDDM
Age at onset	Childhood and young adulthood	Middle and old age
Race	Predominately Caucasians	All races
HLA linkage	Yes	No
Discordance in monozygotic twins	Yes	No
Mechanism	Autoimmune destruction	Unknown
Initial pathology	Insulitis; reduction in β-cell mass	Little or none
Late pathology	Absence of β cells; increase in α and δ cells	Amyloid deposition; fibrosis; β-cell mass normal or moderately reduced
First functional abnormality	Decrease in glucose-stimulated insulin secretion	Decrease in glucose-stimulated insulin secretion

unable to respond to blood glucose increases in excess of normal fasting levels. This would result in the continuation of postprandial hyperglycaemia, the first clinical symptom of diabetes.

Immunoglobulins isolated from the sera of recently diagnosed IDDM patients have been demonstrated to interfere with both beta cell glucose transport (Johnson et al., 1990a) and insulin secretion (Kanutsuna et al., 1983). Dispersed islets, which have been incubated with diabetic antisera, exhibit a reduction in the uptake of 3-O-methyl-D-glucose when compared to normal control sera. From this observation it has been inferred that serum antibodies from diabetic patients will react with either Glut 2 or a putative protein which influences the Glut 2 function.

The levels of Glut 2 are also reduced in the beta cells of animals which have undergone autoimmune destruction of the pancreas. Orci et al., (1990b) have made observations from these animal models at the onset of diabetes and found that only 48 % of the surviving beta cells expressed Glut 2. In addition to this there are many other reports of immunoglobulins from diabetics which inhibit glucose stimulation of insulin release. The presence of anti-Glut 2 antibodies was not determined in these preparations (Kitagawa et al., 1990).

In Type 2 diabetes there is no consistent reduction in the number of beta cells within the islet. A loss of

glucose stimulated insulin secretion at the onset of fasting hyperglycaemia occurs without a parallel loss in the arginine response. Unger, 1991 proposed that this was due to a defective high capacity glucose transporter. NIDDM animal models (inbred colony of a glucose intolerant Zucker fatty rats) have been used (Clark et al., 1983) to examine this hypothesis. This work has documented that in prediabetic rats, the Glut 2 levels are normal but are undetectable in the severe diabetic animals (Johnson et al., 1990c; Orci et al., 1990a). The presence of some highly charged amino acid (amino terminal) residues at the endofacial side of Glut 2 (Newgard et al., 1990), may facilitate an interaction between Glut 2 and the glucokinase enzyme.

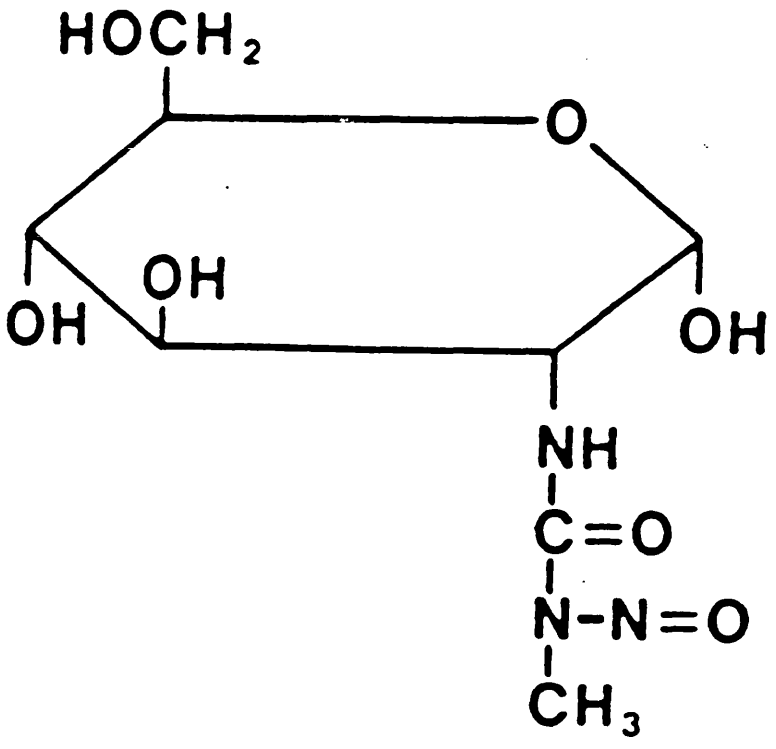
Oka et al., (1990b) demonstrated that in streptozotocin-induced-diabetic rats, the Glut 2 transporter was increased two fold in liver membrane homogenates when these were compared to the normal controls. Upon treatment with insulin both the levels of the transporter and its mRNA were reduced in liver cells. Experimental rats injected with STZ have been demonstrated to develop NIDDM, while at the same time these rats also exhibited a reduced expression of the Glut 2 isoform in beta cells (Thorens et al., 1990a and 1990b).

1.7: A background to the diabetogenic drug streptozotocin

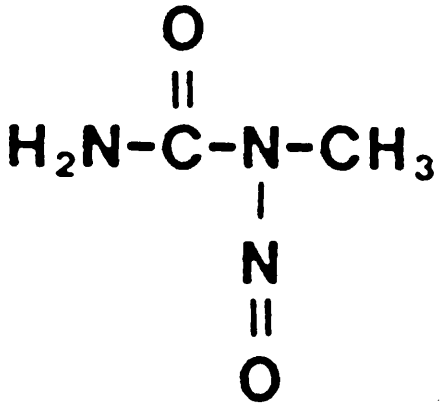
Streptozotocin (STZ) is a broad spectrum antibiotic (Vavra et al., 1960) and an antitumour agent, isolated originally from the bacterium *Streptomyces Achromogenes*. It is a 2-deoxy-D-glucose analogue with the cytotoxic moiety N-nitrosomethylurea (MNU) attached. When STZ is administered to animals it specifically destroys the beta cells and renders the animal diabetic. The structures of both STZ and MNU are shown in figure 1.5.

Schein et al., (1973) studied the diabetogenic activity of STZ in islets and correlated its use with a reduced pyridine nucleotide concentration. STZ was found to inhibit radio-labelled nicotinamide incorporation into the nicotinamide adenine dinucleotide (NAD) molecule. Nicotinamide administration to various experimental animals has been shown to reverse the action of STZ. An observed partial block in NAD synthesis with STZ (Schein et al., 1973) was thought to be due to an inhibition of the polyadenosine diphosphoribose synthetase (Uchigata et al., 1982). Lazarus and Shapiro (1973) demonstrated that NAD partially prevented diabetes. This partial protection effect was possibly due to the poor uptake of the NAD molecule into whole cell islets. STZ and MNU were both observed to produce reduced hepatic NAD concentrations. MNU was also reported to be

Figure 1.5: Chemical structures of Streptozotocin and N-methyl-N-nitrosourea.



Streptozotocin



N-methyl-N-nitrosourea

non-diabetogenic in islets when it was exposed at physiological concentrations (Schein, 1969). These two compounds both exhibited a tissue specific pattern of transport and the uptake of STZ into the islets was reported to be four fold greater than MNU (Anderson et al., 1974). MNU is a nonpolar lipid soluble molecule which will passively diffuse across the membrane. In streptozotocin (STZ), the combination of a glucose moiety and the MNU moiety may have produced a structure which facilitates its recognition by the transporter. Once inside the cell the nitrosomethylurea side chain would reduce the level of intracellular NAD through the lesion of the target cells DNA (Uchigata et al., 1982). This mechanism has been proposed to involve the methylation of the DNA which would then require repair via the key enzyme polyadenosine di-phosphoribose synthetase. This enzyme uses NAD as a substrate and therefore this may account for the lowering of the intracellular NAD concentration. This process will affect the islet cell function, by an inhibition of proinsulin synthesis which may in turn destroy the beta cell (Uchigata et al., 1982; Yamamoto et al., 1981). Evidence which supported the above theory has come from Sandler and Swenne, (1983). These authors performed a series of studies with compounds which were demonstrated to inhibit the synthetase enzyme, while at the same time protecting against the STZ-induced depression of the

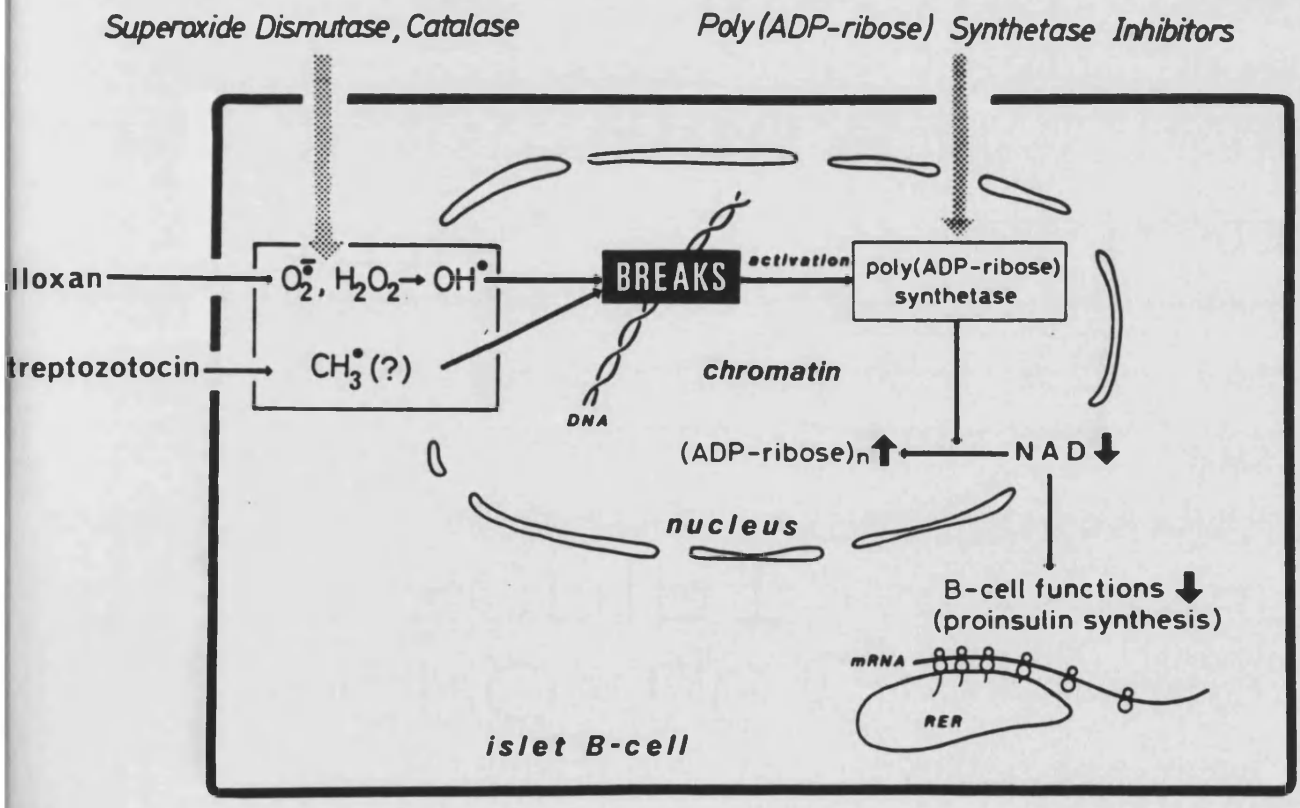
bio-synthesis of insulin. The mechanism for the proposed action of STZ and another diabetogenic agent alloxan (ALX) are outlined in figure 1.6.

STZ has been shown to affect the beta cell in other reversible ways. These functional defects include a decrease in glucose stimulated oxygen uptake, glucose oxidation and insulin secretion. These effects are observed when the beta cell is challenged with D-glucose (Gunnarsson et al., 1974). Such effects have also been seen (but were attenuated) when beta cells were exposed to NMU. Gunnarsson et al., (1974) have suggested that a diminished toxicity with the MNU may indicate that the 2-deoxy-D-glucose moiety is important in facilitating its transport into the cell.

1.7.1: Evidence for a membrane recognition site for the glucose moiety in the streptozotocin molecule

A glucose recognition site on the pancreatic beta cell membrane which recognises STZ has been identified. The effects of STZ have been demonstrated to be inhibited by specific sugar analogues, namely 3-0-methyl-D-glucose and 2-deoxy-D-glucose, but not by alpha-0-methyl-3-0-methyl-D-glucose. Kawada et al., (1987) found that specifically substituted sugar analogues of STZ, such as those with D-mannose and D-galactose instead of D-glucose are nondiabetogenic. The analogue 4,6-0-ethylidene-D-

Figure 1.6: A hypothetical mechanism to account for the protection of islet beta cells against the action of Streptozotocin. As indicated by the shaded lines, superoxide dismutase and catalase may protect against alloxan-induced DNA strand breaks by preventing the formation of OH radicals. Poly (ADP-ribose) synthetase inhibitors may also protect against alloxan or Streptozotocin-induced depression of proinsulin synthesis by the inhibition of NAD degradation (Uchigata et al., 1982).



glucose, which protects against STZ toxicity, has been shown to be a non transported inhibitor. This analogue has also been demonstrated to bind to the extracellular surface of the glucose transporter from human erthrocytes (Barnett et al., 1975), rat adipocytes (Holman et al., 1981) and the blood brain barrier of rats (Dick et al., 1984). These studies provide indirect evidence for the presence of a glucose transporter on the beta cell membrane which transports STZ. A nonmetabolised 3-0-methyl-STZ analogue has also been demonstrated in rats, to be as potent as the parent STZ molecule (Kawada et al., 1986).

Ledoux and Wilson, (1984) reported that an active glucose sensing mechanism may be necessary to enhance STZ cytotoxicity in both normal and neoplastic beta cells. Certain RIN cell lines were found to be resistant to STZ damage compared with beta cells. These RIN cells were also shown to have attenuated levels of glucose-induced insulin secretion. In addition to the above studies, STZ was shown to cause both a dose and time dependent damage of the DNA of this RIN cell line. Similar studies with the MNU molecule in RIN cells demonstrated an attenuation in its diabetogenic activity. Ledoux and Wilson (1984) speculated that STZ entered the cell by the process of passive diffusion, with the overt cell toxicity being avoided in the cells because of a low uptake of the drug. Overall it has been inferred that the effect of the

glucose moiety upon the STZ molecule appears to facilitate its transport into normal beta cells. The glucose moiety is therefore proposed to be important for the selectivity of its action (Anderson et al., 1974).

1.7.2: Toxicity of streptozotocin through the generation of free radicals

The mechanism whereby STZ stimulates the generation of peroxide radicals within the cell, which ultimately leads to the fragmentation of that cells DNA has been investigated by Takasu et al., (1991). These authors proposed that within the target beta cell, diabetes may be induced through the following chemical events.

STREPTOZOTOCIN

⇩

PEROXIDE RADICALS

⇩

DNA FRAGMENTS

⇩

BETA CELL DEATH

When STZ was injected into an animal it appeared to accumulate within the islet very rapidly (Tjalve et al., 1976). To study the formation of free radicals within the beta cell, the spectrographic

technique of electron spin resonance spectroscopy has been used (Nukatsuka et al., 1988). STZ has been shown to increase the electron spin resonance signal resulting from the generation of oxygen radicals. It is known that beta cells contain a high xanthine oxidase activity and a low superoxide dismutase activity. The cytotoxic effects of STZ may therefore be associated with the generation of free radicals (peroxide and oxygen), either directly due to STZ or via stimulation of the xanthine oxidase enzyme.

1.8: Aims of the thesis

1. To perform a kinetic investigation into the D-glucose transport system of specific beta cell lines using 6-deoxy-D-glucose and D-fructose as substrate's. To determine whether the expressed transport system is of the Glut 2 type. To define whether other transporter types are present in these cell lines and to evaluate whether these cell lines constitute an accurate model of the glucose transport properties of the beta cell.
2. To investigate the specificity of the transport protein using the four different cell lines and observe any differences between these systems and other fully characterised transport systems. To look at the relationship between the toxicity of streptozotocin and the specificity of its binding to islet beta cell lines. To work towards an elucidation of how the drug induces

chemical diabetes and also investigate the possibility of a specific glucose recognition site for glucose sensing.

3. To synthesize a radiolabelled analogue of the diabetogenic drug streptozotocin and study the kinetics of its uptake into pancreatic beta cell lines. To establish whether a specific transport protein exists for the drug. A determination of whether streptozotocin utilizes the same transport system as 6-deoxy-D-glucose and D-fructose.

CHAPTER 2 MATERIALS AND METHODS

2.1: Materials

All the non-radiolabelled sugars and streptozotocin were supplied by Sigma Chemical company, Poole, Dorset. Reagents for the organic reactions were supplied by Aldrich Chemical company, Gillingham, Dorset. Ion exchange resins were supplied by BDH Ltd., Poole, Dorset and Whatmann Ltd., Maidstone, Kent. Silica gel-G 60 and silica gel-G 60-F preformed thin-layer chromatography plates were from Merck, BDH, Poole, Dorset. The scintillation fluid was Optiphase which was supplied by BDH. All other reagents were of analytical grade or the highest grade available.

2.1.1: Radiochemicals

3-O-methyl-D-(U-^{14}C)-glucose (295 mCi/mmol), 2-deoxy-D-(-^{3}H)-glucose (6.6Ci/mmol), D-(U-^{14}C)-fructose (304 mCi/mmol), D-(U-^{14}C)-sucrose (300mCi/mmol), D-1-(U-^{14}C)-glucosamine (58.7 mCi/mmol) and (^{3}H)-cytochalasin B (295mCi/ mmol) were supplied by Amersham International U.K. 6-Deoxy-D-(^{3}H)-glucose (8.53 Ci/mmol), was synthesized and supplied by Dr.G.D.Holman.

2.1.2: Buffers

In all buffers the final pH was adjusted with 6M NaOH or HCL where apppropriate. 4-(2-Hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES) supplemented salts buffer was prepared according to the following protocol.
HEPES (10mM), $NaH_2PO_4.2H_2O$ (2.5 mM), NaCl (130 mM), KCl (5mM), $MgSO_4.7H_2O$ (1mM) and $CaCl_2.2H_2O$ (2mM) were all dissolved in 1 litre of deionised water. The buffer was adjusted to a final pH of 7.4.

Phosphate buffered saline (PBS) and calcium magnesium free PBS tablets were purchased from Oxoid, ltd. A PBS buffer was also prepared according to the following protocol. Na_2HPO_4 (12.5mM) and NaCl (154 mM) were dissolved in 1 litre of distilled water. This buffer had its final pH adjusted to 7.2.

A haemolysing buffer was prepared with Na_2HPO_4 (5mM), Ethylene diamine tetra acetic acid (EDTA) (1mM) and 5 µl of Phenyl methoxysulphonalfluoride (PMSF) solubilised in Dimethylsulphoxide (DMSO) (10 mg PMSF: 50µl DMSO). The final volume of this buffer was made up to one litre with distilled water. The final pH of this haemolysing buffer was adjusted to 7.8.

A copper tartrate carbonate mixture was prepared essentially as follows. Sodium potassium tartrate (2%), copper sulphate (1%) and sodium hydroxide (1M) were all

added to distilled water to provide a stock solution. Then the above stock solution was added to a 2% sodium carbonate solution to provide the final buffer.

2.1.3: Cultured pancreatic beta cell lines used

Four cultured beta cell lines were used in the work described in this thesis. The HIT-T15 cell line (1990) was provided by Dr. R. F. Santerre, Boston (MIT), U.S.A. The HITm2.2 cell line (1989) was obtained from Dr. D. Boam, Birmingham Medical School, Birmingham. The RINm5f cell line (1989) was provided by Dr. W. Atkins, University of Aston, Birmingham and the MIN-6 cell line (1991) was provided by Dr. Y. Oka, Osaka, Japan. All of these cell lines were obtained with the original passage number less than 7.

2.1.4: Tissue culture items used

All of the mediums and supplementary materials were supplied by Flow Labs, Irvine, Scotland.

2.2: Methods

2.2.1: Tissue culture procedures. The defrosting and cryopreservation of the cell lines

HITm2.2 and the RINm5f cell lines (passage 5) were maintained in a Rosewell Park Memorial Institute (RPMI) 1640 supplemented tissue culture medium. These cells were grown as a monolayer in 9 cm^3 petri dishes. RPMI 1640 medium was supplemented with 10% heat inactivated foetal calf serum (FCS), 1% L-glutamine (200 mM stock) and a 2% penicillin streptomycin antibiotic stock (5000 IU/ml:5000g/ml). These cell lines at an appropriate density of 7x 10^6 per cryotube were preserved in FCS and DMSO (9: 1 v/v). Each cryotube which contained cells was cooled in the vapour phase of liquid nitrogen with an appropriate slow cooling unit. While the cells were cooled it was important that the temperature decreased at a precise rate of -1^oC per minute until they reached -80^oC. After approximately 20 hours the cryotubes containing the cells were transferred to the liquid phase of liquid nitrogen. Here HITm2.2 and RIN m5f cells were stored at an approximate temperature of -196^oC until they were required for experiments. The HITm2.2 and RINm5f cells were grown in a standard LEEC incubator at 37^oC. This incubator contained a humidified gaseous mixture which was comprised of 95% air/ 5% CO_2.

The HIT-T15 cell line (passage 4) was cultured as a monolayer in a HAMS-F12 complete medium. This medium was supplemented with 10% FCS, 1% L-glutamine (200 mM stock) and 2% of a penicillin streptomycin (5000 IU/ml: 5000g/ml) antibiotic. HIT-T15 cells were cryopreserved following exactly the same slow-cooling protocol described above. The HIT-T15 cell line was frozen in a Hanks balanced salts solution containing 0.5 % lactalbumin, new-born calf serum (NCS) and DMSO (8: 1 v/v). These cells were grown and maintained in a LEEC incubator at 37^{o}C. The atmosphere inside the incubator was a mixture of 95% air and 5% CO_2. HIT-T15 cells reached confluence at a density of 5x 10^6 cells per dish. These cells were preserved at this density and then the confluent cells were split (1: 2) and grown at a lower density for a time period of 5 days.

The MIN6 cell line (passage 6) was grown and maintained in a Dulbeccos Modified Eagles medium (DMEM). This medium was supplemented with 20% FCS, 1% L-glutamine (200mM stock), 2% of a penicillin streptomycin stock antibiotic (5000 IU/ml: 5000g/ml) and 0.7 % 2-mercaptoethanol. These cells were grown at 37^{o}C in an atmosphere which contained 95% air and 5% CO_2. MIN-6 cells were cryopreserved after reaching approximately 70% confluence using the same protocol which was used to store the HITm2.2 and RINm5f cell

lines. MIN-6 cells were also preserved in a FCS: DMSO (9:1 v/v) mixture.

2.2.2: The growth and passage of the cell lines

The HIT-T15, HITm2.2 and RINm5f cell lines were rapidly defrosted by the addition of 2 ml of the appropriate growth medium to a cryotube. The contents of the cryotube were added to the appropriate culture medium to make up a final volume of 20ml. After this, the cells were centrifuged for 2 minutes at 1500 RPM in a bench centrifuge. The supernatant was then removed and the cell pellet was resuspended in an aliquot of medium. The cells were then vortexed gently and aliquoted into 9 cm^3 tissue culture plates, before they were grown in 10 ml of the appropriate culture medium.

All of the three cell lines had their medium replaced after approximately 48 to 72 hours. After four to five days when the cultured cells reached confluence, they were re-seeded and then maintained at a lower cell density (HITm2.2 and RINm5f cells between 1: 5 and 1: 7 and the HIT-T15 cells no more than 1: 3). The medium was discarded and the cells were then pre-washed with 10 ml of a sterile PBS buffer. After this buffer had been removed, 0.5 ml of a trypsin EDTA solution (0.25% v/v) was added at $37^{o}C$ for approximately 1 minute (HIT-T15 cell line, a maximum of 5 minutes incubation

with trypsin at 37^{o}C). The cells were then passaged gently and resuspended in 20 ml of the appropriate growth medium. Following this the cells were centrifuged at 1500 RPM for 5 minutes in a bench centrifuge. After centrifugation the cell supernatant was subsequently discarded and the remaining cell pellet was quickly resuspended in an appropriate volume of growth medium. The cells were then mixed gently and reseeded into sterile tissue culture dishes (10 ml of medium per dish).

The MIN6 cell line was grown and maintained under slightly different conditions. These cells were initially defrosted at 37^{o}C, with an appropriate volume of the DMEM growth medium. The cryotube contents were then added to 20 ml of medium and centrifuged at 900 RPM for 5 minutes. The pellet was then resuspended in 5 ml of medium and the cells were grown in a 25 cm^3 sterile flask for 24 hours.

MIN6 cells had their medium replaced every 48 to 72 hours and they achieved confluence in one week. After this time period, they were subcultured and grown at a lower seeding density as described below. The medium was removed from the flask and the cells were washed twice with sterile PBS (2x 5ml washes). This wash buffer was discarded and 0.5 ml of a trypsin EDTA (0.25% v/v) solution was added to the cells. The flask was incubated at 37 ^{o}C for 3 minutes before being tapped gently to encourage cell detachment. This was followed by a resuspension of the cells in 10 ml of growth medium.

During this time, care was taken not to produce a single cell suspension as it was reported that only aggregated cells would grow successfully.

The MIN-6 cell suspension was spun at 900 RPM for 5 minutes. After this centrifugation, the supernatant was discarded and the cells were resuspended in 5 ml of medium. These cells were then seeded at a lower density and were grown in 25 cm^3 flasks (1:5 split) or 9 cm^3 tissue culture dishes (1:2 split). The cell density was determined by using a standard graded haemocytometer and a trypan blue cell stain. Cells were then examined with a phase contrast microscope.

2.2.3: The preparation of erythrocyte cell membranes

The preparation of erythrocyte cell membranes was performed essentially according to the protocol described by Gorga and Leinhard, (1981). To prepare the erythrocyte cell membranes, approximately 100 ml of human blood was divided between two 250 ml centrifuge tubes. These were equally balanced with PBS buffer. After centrifugation at 4,500 RPM for three minutes the white cell layer and remaining supernatant were carefully removed. The tubes were then refilled and balanced with PBS and the cell pellet was then resuspended and centrifuged again.

The supernatant was removed and the remaining cells were divided into six 250 ml centrifuge tubes. Each tube

was filled with ice cold haemolysing buffer and left on ice for around 20 minutes. Following centrifugation at 11,000 RPM for 20 minutes, the supernatant was removed and the cells were washed with the haemolysing buffer and centrifuged once again at 11,000 RPM for 20 minutes. This washing procedure was repeated three to five times until the supernatant was clear in appearance. The membranes were finally washed with 5 mM phosphate buffer and spun finally at 11,000 RPM for 20 minutes.

After this, both the supernatant and the cloudy membrane preparation were discarded. The red cell pellet which remained was solubilised in PBS and divided into 1 ml aliquots. These membrane aliquots were stored at $-70^{o}C$ and a standard Lowry protein determination was performed (Lowry et al., 1951).

2.3: Sugar transport methods and uptake protocols

The following transport methods were a modification of Eliam and Stein, (1974).

2.3.1: 2-deoxy-D-glucose and 3-0-methyl-D-glucose transport into the HITm2.2 cell line. The effects of two standard transport inhibitors, cytochalasin B and phloretin upon uptake into the HITm2.2 and RINm 5f cell lines.

All of the solutions and buffers were preincubated at 37^{o}C before the assay was started. The HITm2.2 and RINm5f cells (passage 10-20) were seeded at 0.15x 10^{6} cells per ml into six 4 well (1ml volume) tissue culture dishes. After 48 to 72 hours the cells reached confluence at 2x 10^{6} cells per ml. The culture medium was then carefully removed by a gentle aspiration. The cells were washed with PBS (2ml per well) before the uptake of the sugar was assayed. Triplicate samples were analysed and each well was pre-treated for 2 minutes with 50 µl of inhibitor (20 µM final cytochalasin B and 100 µM final phloretin concentrations). This inhibitor was added carefully to cover the entire surface of the cells.

The assay was started by the addition of 50 µl of the radiolabelled sugar (either 2-deoxy-D-glucose or 3-0-methyl-D-glucose), at a final sugar concentration of 50 µM (0.1µCi/ ml well). The sugars were incubated for

varying time periods for the time course experiments. For time points of 10 seconds or less a metronome set at two beats per second was used. Transport was terminated by the addition of 3 ml of a 0.1 mM phloretin stopper solution which was added carefully down the side of the well. Care was taken not to disturb the cell monolayer. The solution was removed by gentle aspiration before a second phloretin wash was performed. After both washes, all the visible adhering droplets were removed and the wells were allowed to dry in air for 1 minute. Finally 0.5 ml of a 0.1 M NaOH solution was added to each well to lyse the cells. The cell lysates were then aliquoted into scintillation vials (0.45 ml lysate: 5ml scintillant) for the CPM'S to be determined by a liquid scintillation counter. Zero-time counts were subtracted from all subsequent uptake values. These zero time values were estimated by addition of the radiolabelled sugar and phloretin solution simultaneously. Isotope equilibrium values were obtained by incubation of the cells for 120 to 600 seconds with the radiolabelled sugar. Using the equilibrium value the fractional filling of the cells is calculated as follows:

$$f = \frac{CPM_t - CPM_o}{CPM_i - CPM_o} \qquad \text{equation a}$$

Here f is the fractional filling, CPM_t are the counts per minute at time t, CPM_i are counts per minute at isotope equilibrium and CPM_o are the counts per minute at zero time.

2.3.2: 6-deoxy-D-glucose and D-fructose transport into the HITm2.2 and RINm5f cell lines

HITm2.2 and RINm5f cells (passage 15-25) were seeded at 0.2x 10^6 cells per ml into 9 cm^3 tissue culture dishes. These cells reached confluence in 5 to 7 days. After this time period the cells were first detached from the dishes and the following uptake assay was performed on cells in suspension. The culture medium was removed and the cells were washed with PBS (10ml, 37^o C) twice before being subjected to a gentle aspiration. Then 5 ml of a detachment buffer (1mM EDTA in PBS) was added for 3 minutes at 37^{o}C. After this time the cells were removed by gentle passage and the contents were then transferred to a universal tube. A standard cell count was performed before the cell suspension was spun at 1500 g for 3 minutes in a bench centrifuge. The cell pellet was then resuspended in a HEPES (glucose free) buffer and respun

to remove any residual EDTA. Following this the cells were then seeded at 10x 10^7 cells per ml. At this stage, and during the assay, it was important to periodically mix the cells to maintain an even suspension.

An appropriate number of assay tubes were then prepared. Each contained a final assay volume of 30µl. Each assay consisted of 5 µl of the competing sugar or buffer. The inhibitory sugar was diluted from a six times stock at a convenient concentration range. In addition to this 5 µl of radiolabelled 6-deoxy-D-glucose at 100uM final concentration (0.2µCi per tube) was also carefully pipetted to form a 10 µl droplet at the bottom of each tube (5µl 6-deoxy-D-glucose + 5µl inhibitory sugar or buffer). Transport at 22^{o}C was initiated by addition of 20 µl of suspended cells to each centrifuge tube. Here accurate pipetting was performed and care was taken to uniformly add the cells to the radiolabelled sugar precisely at the start of timing. For accurate manipulation no more than 3 tubes were processed at any one time.

Sugar uptake was stopped by the addition of 2 ml of a 0.1 mM ice cold phloretin stopper solution. Each tube was then spun at 1500 g for 2 minutes in a bench centrifuge. The supernatant was then carefully removed by very gentle aspiration, which ensured that all of the adhering droplets which contained radiolabelled sugar were removed. This was followed by a second wash with the

phloretin stopper solution. Each tube was dried to remove any radiolabelled buffer without disturbing the cell pellet. After all uptake estimations had been completed, the cell pellets were lysed by a resuspension in 0.5 ml of a 0.1 M sodium hydroxide solution. The cell lysates were incubated at 20^{o}C for 30 minutes. These were then dispensed into scintillation vials (0.45ml lysate: 5ml scintillant) for the CPM'S to be determined by liquid scintillation.

Zero-time counts were subtracted from all subsequent uptake values. These were determined by addition of both radiolabelled sugar and the phloretin solution simultaneously. Isotope equilibrium values were obtained by incubation of cells for 60 to 120 seconds with labelled 6-deoxy-D-glucose. Using the equilibrium value the fractional filling of the cells was calculated as in equation a.

The inhibition data is analysed by a plot of v_o/ v against the competing sugar concentration (I). Here v_o is the uninhibited rate of sugar entry, v is the inhibited rate and V is the osmotic volume (equation b_1). v is expressed in units of concentration sec^{-1} and is calculated as follows:

$$v_o = \frac{-1 \ln (1-f).s_o}{V \quad t} \qquad \text{equation b}$$

equation b_1

V= Osmotic volume = $\frac{293 + \text{Inside sugar concentration}}{293 + \text{Outside sugar concentration}}$

These equations assume that the backflux is non-saturable. From equation b, f is the fractional filling, s_o is the initial substrate concentration outside and t is the incubation time in seconds. For single time point assays an incubation time of ten seconds was used to calculate the uptake rate. This represented a fractional filling of approximately 40%.

For the transport of D-fructose the same method was used as described in section 2.3.2.

2.3.3: 6-deoxy-D-glucose, D-fructose and streptozotocin transport into the HIT-T15 and MIN-6 cell lines

This assay was modified from that described in section 2.3.2. HIT-T15 and MIN6 cell lines were grown in 9 cm^3 dishes until they reached confluence at around five days. The cells were reseeded (1:3 split) into 3 cm^3 dishes and

grown for four days before they reached confluence at $3x\ 10^6$ cells per dish on the 5^{th} day.

Transport was performed in 3 cm^3 tissue culture dishes. Before the assay was started the cells were washed twice with 3 ml of a calcium and magnesium free PBS. After each wash the PBS was removed by a gentle aspiration. Each assay was performed in triplicate using HEPES glucose free buffer. Sugar uptake commenced with the addition of 150 µl of a 100uM final concentration of 6-deoxy-D-glucose (0.2µCi per dish). In addition to the 6-deoxy-D-glucose, 75 µl of a competing sugar for the kinetic experiments or HEPES buffer was included for the time course experiments. For incubation times of less than 10 seconds a metronome set at two beats per second was used for timing purposes. Uptake was stopped by the addition of 3 ml of a 0.1 mM phloretin stopper solution. The phloretin solution was removed by careful aspiration and each dish was allowed to dry before the cells were washed again with a phloretin solution. Finally all the dishes were allowed to dry for 5 minutes. No more than three dishes were processed at any one time and each dish was removed from the 37°C incubation only when it was required. After all the uptake experiments were completed the cells were lysed by a resuspension in 1 ml of 0.1 M NaOH and allowed to stand for 30 minutes at 22°C. The lysates were then added to scintillation vials (0.95 ml lysate: 5 ml scintillant) for the CPM'S to be

determined. The uptake of the sugar label was expressed as both a fractional filling and an initial velocity value as outlined in section 2.3.2.

Studies on the uptake of D-fructose were carried out using the same method as described in 2.3.2. In this case, a 100 µM final concentration of D-fructose (0.2µCi/ dish) was substituted for 6-deoxy-D-glucose. Similarly radiolabelled streptozotocin uptake and kinetics studies were performed as in 2.3.2. The modification here was that a 100 µM final concentration of streptozotocin (0.2µCi/ dish) was added to the cells in a final assay volume of 100 µl.

2.3.4: Investigation of streptozotocin toxicity in the HITm2.2 and RIN m5f cell lines

Essentially this method was a modification of that described in section 2.3.2. HITm2.2 and RINm5f cells were seeded at 0.15x 10^6 cells per dish into 9 cm^3 sterile culture dishes and were exposed to STZ. 6-deoxy-D-glucose transport assays were performed in suspension as outlined in section 2.3.2.

Experiments were also carried out with the phosphorylated analogue 2-deoxy-D-glucose. These studies performed on HITm2.2 cells, were an investigation upon the effect of the long term incubation of streptozotocin and its effect upon 2-deoxy-D-glucose transport. Here STZ

was incubated at a final concentration of 5 mM for 48 hours. After this time the uptake of 2-deoxy-D-glucose was assayed. HITm2.2 cells (passage 15) were seeded at 0.15x 10^6 cells per ml into 4 well tissue culture (1 cm^3) dishes. The cells reached confluence in 48 to 72 hours at 2x 10^6 cells per dish. After 24 hours the growth medium was removed and the cells were washed three times with 3 ml of sterile PBS. A minimal glucose culture medium (containing glucose at 5 mM) was added to the cells. A final concentration of 5 mM STZ was added. This solution was sterile filtered from a convenient stock solution. Transport of 2-deoxy-D-glucose was performed as outlined in section 2.3.1. and the results were expressed as CPM against time.

An inhibition constant (K_i) for streptozotocin inhibition of 2-deoxy-D-glucose transport in the HITm2.2 cell line was obtained. This method was essentially the same as described in 2.3.1 but various concentrations of STZ from 0 to 50 mM were substituted for phloretin and cytochalasin B.

2.4: An investigation of the binding of streptozotocin to the erythrocyte cell membrane

2.4.1: The inhibition by streptozotocin of cytochalasin B binding to the erythrocyte membrane

All of the buffers and the tubes were maintained at 4 ^{o}C during the course of the experiment. At the start of the experiment the tubes were set up in duplicate with a total assay volume of 230 µl. This included STZ at 0, 1, 2, 5, 10 and 20 mM. The assay also contained a radiolabelled cytochalasin B stock solution, which included 2 mM cytochalasin E (final concentration) and a 1 mM final concentration of cytochalasin B (0.2µCi/ ul). Each time the difference in the final volume was made up by the addition of 5 mM phosphate buffer, adjusted to a final pH of 8.

The reaction was started by the addition of 180 µl of erythrocyte membranes (1mg/ ml) and the contents were then incubated at 4^{o}C for three minutes. After this time period the tubes were spun in a refrigerated microfuge at 20,000 g for one minute. Then two 50 µl aliquots of the supernatant were counted in 8 ml of scintillation fluid. A 10 µl aliquot of the cytochalasin B stock was also counted to obtain the total counts per minute. The inhibition of cytochalasin B binding by STZ was expressed

as free cytochalasin B / bound, against the free STZ (inhibitor) concentration.

A plot which analysed the streptozotocin inhibition of the cytochalasin B binding to the erythrocyte cell membrane was also performed according to the following protocol.

Here a series of tubes were set up in duplicate with an appropriate range of cytochalasin B concentrations with and without a final concentration of 4 mM STZ. A dual labelled stock which contained a 2 mM final concentration of cytochalasin B (0.2µCi/ µl) and radiolabelled sucrose (0.2µCi/ µl), were added to each tube. The total assay volume of 40 µl was adjusted accordingly with a 5 mM phosphate buffer adjusted to a final pH 8.

A 180 µl aliquot of a 1mg/ ml membrane suspension was added to each tube, mixed and allowed to stand at $4^{o}C$ for three minutes. Each tube was then spun at 20,000 g for one minute. Then, two 25 µl aliquots of the supernatant were counted in 8 ml of scintillation fluid. Two vials which contained the 20 µl aliquots of the dual labelled stock were also counted. The latter gave the total separate (^{3}H) and (^{14}C) CPM's. The results were expressed in a graphical format outlined in chapter 3. All of the protein concentrations were determined by using the Lowry method (Lowry et al., 1951).

2.4.2: An investigation of the reversibility or irreversibility of streptozotocin inhibition of cytochalasin B binding

A series of tubes were set up and incubated at 37°C for a range of time points (30 minutes to 18 hours). One series of tubes contained a final concentration of 10 mM STZ while the other contained 0mM STZ. In this assay each tube contained a final concentration of erythrocyte ghost membranes at 1 mg per ml suspended in PBS buffer. The final assay volume was 210 µl and the pH was adjusted to 8.

Following the incubation with membranes each tube was spun at 20,000 g for one minute in a refridgerated microfuge. The supernatant was then removed and the membranes were resuspended in a 210 µl volume of phosphate buffer (pH 8). Following this, 10 µl of a 1 mM final cytochalasin B solution was added to a tube which contained no inhibitor. This control tube was incubated for a zero time period.

A 10 µl aliquot of a radiolabelled cytochalasin B stock (0.2µCi/ tube) which contained final concentrations of 2 mM cytochalasin E and 1 mM cytochalasin B, was added to each tube. The tubes were then incubated on ice at 4°C for 3 minutes. Each tube was spun in a refrigerated microfuge for 1 minute at 20,000 g. Then two 50 µl aliquots of each supernatant were counted in 8 ml of

scintillation fluid. A 10 µl aliquot of the stock cytochalasin B label was taken for a total CPM determination. A standard protein determination was also performed (Lowry et al., 1951).

2.5: The synthesis of radiolabelled streptozotocin

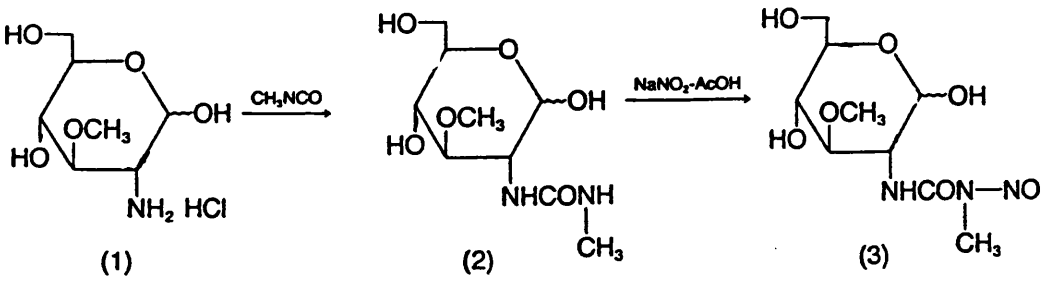
2.5.1: The chemical synthesis of labelled streptozotocin

The synthesis of labelled streptozotocin ((^{14}-C) 2-deoxy-2-({ [methylnitrosoamino) carbonyl} amino]-D-glucopyranose) consists of two main stages illustrated in the following scheme:

(1) = D-Glucosamine hydrochloride

(2) = 2-(3-Methylureido)-2-deoxyglucopyranose (GU)

(3) = 2-Deoxy-2-{[(methylnitrosoamino)carbonyl]amino} -D-glucopyranose (streptozotocin)



The synthesis of STZ was based upon a modified method of Kawada (et al., 1986). It was not clear from the communication whether these authors used commercial

N-methylisocyanate (MIC) for the synthesis. From enquiries made to a number of chemical companies MIC synthesis had been discontinued for the following reasons. It was unstable during transport, toxic when produced on a relatively large scale and also it was not commercially viable. The purity and stability of MIC was important for the formation of the final STZ yield. MIC synthesis was performed with many modifications on two main protocols which are outlined below: (In both cases MIC was synthesized initially on a small scale and scaled up when the optimum conditions had been established).

1. The synthesis of N-methylisocyanate from triphosgene and N-methylamine.
2. The synthesis of N-methylisocyanate from the distillation of dichlorodiphenylsilane and sodium azide.

Details of both methods chosen for the synthesis are as follows. Method 2 was the most successful protocol in terms of the final MIC yield. This method was a distillation process with a well defined endpoint. A measurable quantity of MIC was produced using this method. Method 1 relied on a chemical transformation where a molecule of phosgene would react with N-methylamine to produce MIC.

2.5.2: The distillation of N-methylisocyanate

A two part reaction scheme for the distillation is shown below:

Part 1.

Sodium Azide + Dichlorodiphenylsilane

↓

Trimethyltriazosilane

Part 2.

Trimethyltriazosilane + Acetic Anhydride

↓

N-Methylisocyanate + Nitrogen

Methylcarboxytrimethylsilane

The distillation of N-methylisocyanate was carried out as described in a translation of a method outlined by Kricheldorf, (1972). The MIC was synthesized on a number of occasions. It was found that a suitable yield was achieved using a 12 mmole ($1/50^{th}$) scale. Then a further full scale synthesis was performed. The final yield at this reduced scale was approximately 20% (250µl, 0.24g),

with the amount being increased upon scaling up the distillation.

Dichlorodiphenylsilane (146g, 0.6 moles) was stirred with 1.3 moles of sodium azide in 500 ml of dry quinoline for 10 hours at 120^{o}C. Before the addition of the anhydride the quinoline was heated to 100^{o}C to remove any excess water which may have accumulated. Then, the flask was allowed to cool to 22^{o}C. 1 mole of acetic anhydride was added to the cooled solution which reacted exothermically. The mixture was then heated by oil immersion until nitrogen gas was produced at 70^{o}C. The reaction was controlled by regulation of the temperature so that it continued to its end in approximately 30 minutes. The temperature was only allowed to exceed 100^{o}C for a short time period at the very end of the reaction.

The isocyanate was distilled off through a modified angular distillation head and collected in a flask. The anhydride was converted into the isocyanate with a reported 95 % approximate yield (Kricheldorf, 1972) and therefore the end product yield was dependent on both the distillation and the batch size. Initially the N-methylisocyanate was distilled on a small scale and to ensure a maximum purity the acetic anhydride was redistilled on every occasion. The best yield of MIC which was obtained was 40% (25 ml, 24 g). This distillation was then performed on a number of occasions

to obtain a sufficient quantity of the MIC. The MIC was stored in a desiccator (in the dark) for no more than 24 hours.

Some important factors which were crucial to the production of distilled MIC as the actual yield was only 40% are outlined below. It was important to immerse the reaction vessel at the correct angle in the oil bath. This encouraged a suitable flow rate of MIC which was finally collected. Other important components were the reflux time of the dichlorodiphenylsilane with the sodium azide. Although the recommended time was 10 hours, it was found that an increased reflux time of up to 18 hours or even longer increased the yield of the end product. The Claisen distillation head was constructed with a large-diameter which was reduced towards the condenser. Such alterations minimised the amount of MIC which was lost between the top of the condenser and the collecting vessel.

2.5.3: The synthesis of N-methylisocyanate from triphosgene

The alternative method for the synthesis of N-methylisocyanate which was investigated was based on triphosgene. This was provided by Aldrich International. This method was closely coupled to the formation of STZ during the initial attempts with this method. The reaction for the formation of the MIC is described below:

Methylamine + Triphosgene

Solvent

MIC + Hydrochloric acid + Carbonyl chloride

The synthesis of the N-methylisocyanate was carried out using the following method. 0.12 mmol of methylamine (8.1 mg) was dissolved in 20 µl of solvent (2 mmol solvent: 1 mmol methylamine), before 0.04 mmol (12 mg) of triphosgene was added. The reaction then proceeded through to completion with some modifications.

Several procedures for the synthesis of N-methylisocyanate were attempted as outlined in the following reaction schemes. These summarised the main conditions and reagents that were used. At first the MIC was synthesized separately. The appropriate solvent

systems for both the methylamine and the triphosgene are shown in a flow chart format. These trials were performed at 4°C and at -10°C using non radiolabelled glucosamine. Following the most successful method the yield of MIC was determined by using an aliquot of radiolabelled glucosamine (1μCi). This was achieved by its addition at the start of the reaction. The reaction was allowed to proceed through to the urea stage. The formation of the urea was determined by thin layer chromatography, which exploited two types of solvent system. A quantitative estimation of the formation of GU was determined by the division of a tlc plate into ten equal portions. Each section of the plate was then counted in 5 ml of scintillant by liquid scintillation (Packard counter). This procedure enabled the quantity of radiolabelled GU to be determined.

A) Methylamine + Triphosgene

solvent

pyridine ↓ performed at 4 and -10°C

N-methylisocyanate + Glucosamine hydrochloride

↓ 4°C and 2 hr's.

under stirring

2-(3-methylureido)-2-deoxyglucopyranose

B) Methylamine + Triphosgene
pyridine Dichloromethane
4 and -10°C
N-methylisocyanate + Glucosamine hydrochloride

C) Methylamine + Triphosgene
N-methylmorpholone Dimethylformate (DMF)
4 and -10°C
N-methylisocyanate

D) Methylamine + Triphosgene
Acetonitrile added portion wise
4 and -10°C
N-methylisocyanate

E) Methylamine + Triphosgene
Aqueous Dichloromethane
Glucosamine free base
N-methylisocyanate

Several problems were encountered with methods A) to D). When these methods were performed, no product could be readily identified or purified. This is discussed further in chapter 4. Method E was the most successful method and

is described in detail below. In all of the above flow charts the MIC was synthesized separately at first before it was added to the glucosamine. In method E the glucosamine free base was included together with the other reagents.

2.5.4: The synthesis of radiolabelled 2-(3-methylureido)-2-deoxy-D-glucopyranose (GU) using the triphosgene method

The synthesis of GU is described below. 100µl of D-glucosamine hydrochloride (0.1mmol, 20 mg) and radiolabelled D-glucosamine (1µCi/ 0.1 mmol) were passed through an Amberlite OH^- resin (IRA 93, std. grade) exchange column. This column contained 400mg of the cationic resin. The column was washed again with 100ul of distilled water. A total of 200 µl of the glucosamine free base eluant was collected and concentrated to a minimum volume.

The free base was then stirred in a quick fit flask at 4^oC. 0.12 m moles of triphosgene (35 mg, 50 µl) solubilised in dichloromethane was added portionwise to the mixture. Then aqueous N-methylamine (8.1 mg, 0.12 moles) was added dropwise and the whole solution was stirred at 4^oC for approximately two hours.

The solvents were then removed under nitrogen. Formation of the GU was assessed by thin layer chromatography (tlc) on preformed plates. The solvent

system was Acetonitrile: Acetic acid: Ethanol: water (13:1:2:4) which was finally changed to Ethyl acetate: Methanol: water (5:2:1). It was found that a better separation of the endproduct was achieved with the latter system. This latter solvent system was retained for all of the subsequent experiments.

The residual solution was dissolved in 130 µl of 1 M acetic acid before 0.12 mmol of sodium nitrite (8.5 mg) was added portion wise at 4^{o}C over a 30 minute period. After this time 26 µl of 10% (1M) hydrochloric acid was added dropwise and the whole mixture was stirred at 4^{o}C for approximately 1 hour. At this stage the formation of the final product was assessed by neutralising 5 µl of the endproduct with Amberlite monobed resin MB1 (analytical grade). This synthesized product was set against commercial STZ (Relative front (Rf) of approximately 0.2) using the appropriate solvent system. The reaction mixture was saturated with 5ml of a saturated ammonium sulphate solution (pre warmed at 37^{o}C) and extracted with two 9ml portions of ethyl acetate: tetrahydrofuran (1: 8).

The organic layer was then dried over magnesium sulphate and concentrated by rotary evaporation. A 50 µl aliquot was analysed by tlc. The tlc plate was sectioned into ten equal parts and counted in a liquid scintillation counter (1 tlc plate portion: 5 ml scintillant). The final yield was calculated at approximately 10 % (2 mg, 0.1µCi).

2.5.5: The synthesis of radiolabelled streptozotocin from distilled N-methylisocyanate

To optimise the reaction conditions to ensure a maximum yield of labelled STZ, the synthesis of GU was performed on a small non-labelled scale. This was followed by the synthesis of radiolabelled STZ. The full scale synthesis of radiolabelled STZ was performed twice (48µCi in total; 24µCi per synthesis) according to the modified method of Kawada (et al., 1986).

Several procedures for the synthesis of GU were carried out as indicated by the reaction schemes below. Each time the formation of GU was illustrated by its distribution upon a tlc plate. The solvent system was Ethyl acetate: Methanol: water (5: 2: 1).

A), B) and C)

Glucosamine + N-methylisocyanate

(free base) Temperature

Time A) $22^{o}C$

2 hours ↓↓ B) $4^{o}C$

C) $-10^{o}C$

2-(3-methylureido)-2-deoxy-D-glucopyranose

D), E) and F)

Glucosamine + N-methylisocyanate

(free base) Time

D) 1/2 to 1 hours 22°C

↓↓ E) 2 to 4 hours/ 4°C

F) 4 hours plus/ -10°C

2-(3-methylureido)-2-deoxy-D-glucopyranose

Overall method E was determined to be the most successful. This was assessed by qualitative tlc. The tlc plates were charred with concentrated sulphuric acid and an Rf value of 0.55 was calculated for the end product. After 4 hours or longer the GU was seen to decompose, whereas up to 2 hours some of the glucosamine still remained.

Several procedures were also carried out on the GU leading to the synthesis of STZ. These procedures are indicated in the following reaction schemes. Common parts of the reaction schemes have been combined centrally for simplification. From the first scheme below, both of the methods A) and B) have an ammonium sulphate saturation and a magnesium sulphate/ silica gel column step.

A) B) Glucosamine Urea

A) 1M Acetic acid
$4^{o}C$ portion wise addition
30 minutes stirring
10% Hydrochloric acid
dropwise addition
$4^{o}C$ 1 hour stir

B) 1M Acetic acid
$22^{o}C$ one amount
15 minutes stirring
10% Hydrochloric acid
one addition
$22^{o}C$ 1 hr. stir

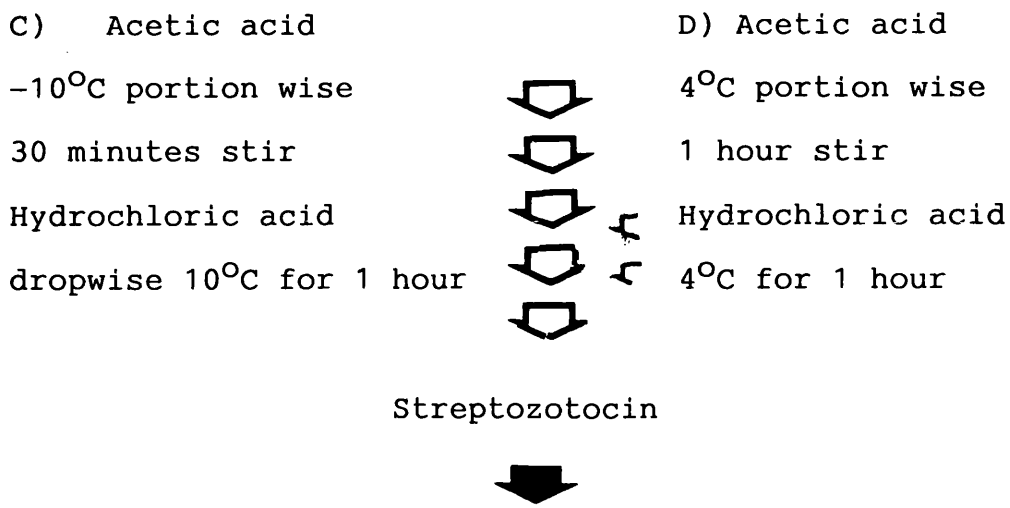
A) B) Ammonium Sulphate saturation
twice extracted with
Ethyl acetate: Tetrahydrofuran (THF)
(1: 8)

A) B) Organic layer dried over
Magnesium Sulphate

(Streptozotocin)

Purified by silica gel chromatography
Ethyl acetate: Methanol: Water, 7: 2:1

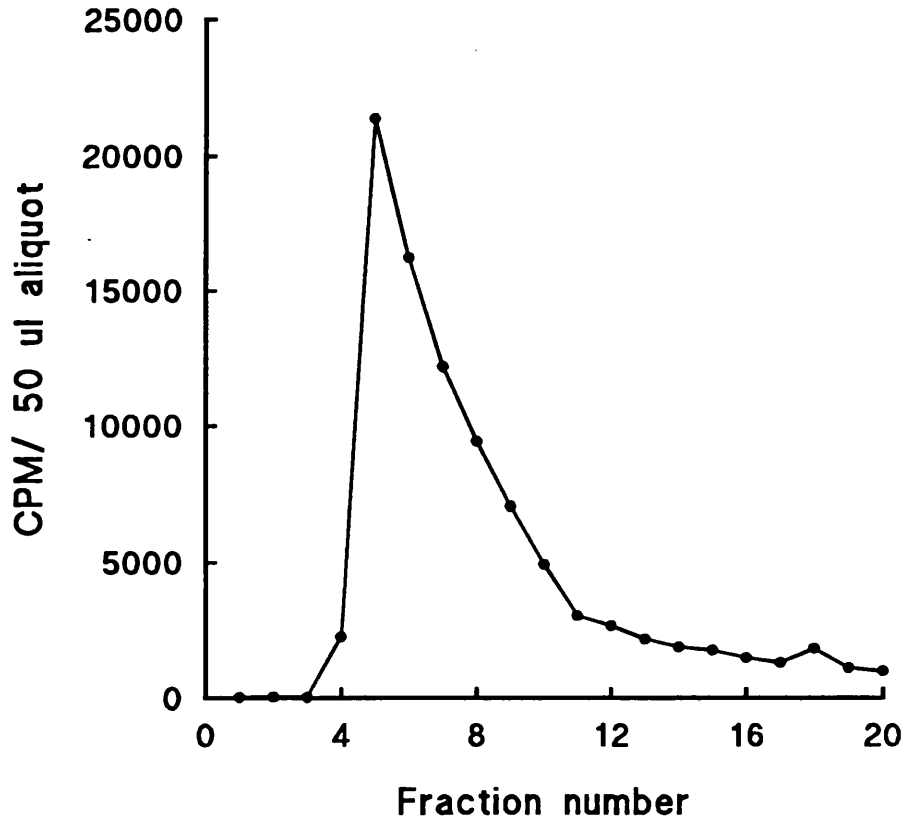
C) D) Glucosamine Urea



The preferred method was estimated to be D. The two methods D and E were combined and are outlined below.

D-Glucosamine hydrochloride (0.025 mmol/ 5 mg) and radiolabelled D-glucosamine hydrochloride (24 µCi per synthesis) were both solubilised in 2 ml of distilled water. This solution was then passed through an Amberlite OH^- resin (IRA 93 standard grade, 0.7 g resin/ mmol glucosamine hydrochloride) column which contained 100 mg of cationic resin. Subsequently, 2 ml of the glucosamine free base mix was eluted through the column which was followed by a second 2 ml wash with distilled water. The total elutant was collected and concentrated to a final minimum volume of 100 ul.

Figure 2.1: Elution profile calculated from fraction aliquots (50µl) of the final product in the synthesis of D-streptozotocin. The fractions corresponding to the large peak were pooled, concentrated and aliquoted. The product was D-1-^{14}C-streptozotocin. The solvent system was Ethyl acetate: methanol: water, (5: 2: 1).



N-methylisocyanate (10mg/10 µl/0.17 mmol) was added to the glucosamine free base at 4°C under stirring for a time period of 2 to 3 hours. After this, the formation of the urea derivative was verified by thin layer chromatography on pre-prepared (Merck) silica gel G 200 plates (solvent system, ethyl acetate : methanol : water; 5 : 2 : 1).

The urea was then dissolved in 33 µl of a 1M (15 %) acetic acid solution and 25 µl of an 80 mg/ ml (0.03 mmol) sodium nitrite stock was added portion wise at 4°C over a 30 minute period.

Finally, 6.5 µl of a 1 M hydrochloric acid solution was added dropwise and the whole mixture was stirred for 1 hour at 4°C. This produced the end product (60% yield, 3 mg) after separation on a silica gel G 200 column with the appropriate ethyl acetate : methanol : water (5 : 2 : 1 v/v) solvent system. The progress of STZ formation was assessed by neutralising a small portion of the endproduct with Amberlite monobed resin (MB1 analytical grade, 1 ml of 1 M HCL to 0.7 g of the resin). The final product was then run against commercial STZ which was the highest grade available.

The product was applied to a silica gel G 200 column and the appropriate number of fractions were collected (50x 1 ml). An aliquot of each fraction was analysed to determine the position of product elution. From figure 2.1 fractions 4 to 10 were concentrated, freeze dried and

stored at -20°C (7x 4μCi aliquots). The final combined yield of radio-labelled STZ from two experiments approximated to 28μCi (58%).

CHAPTER 3 RESULTS OF A CHARACTERISATION OF THE HEXOSE TRANSPORT PROPERTIES OF FOUR PANCREATIC BETA CELL LINES

All of the following graphical plots in chapter 3 are representative examples of two or more experiments. The estimation of the experimental error of each data point has been determined by calculating the mean and standard deviation of triplicate points. Estimations of experimental error for the kinetic and transport data are expressed as the standard error. Where these are given the standard error was calculated from the regression of values obtained in three or more experiments.

3.1: Sugar transport into the HITm2.2 cell line

3.1.1: The transport of 2-deoxy-D-glucose and 3-O-methyl-D-glucose and the effect of two transport inhibitors phloretin and cytochalasin B on sugar uptake

The phosphorylated glucose analogue 2-deoxy-D-glucose and the non-metabolised analogue 3-O-methyl-D-glucose were both used in separate experiments with the HITm2.2 cell line to perform some initial transport studies. These early assays were performed with HIT cells still attached to the tissue culture dish surface. HITm2.2 cells were initially used to optimise the assay because it was found that at a low passage number they formed a stable

monolayer. This provided a stable transport assay system to begin to characterise sugar uptake into these cells. A time course experiment performed with 3-0-methyl-D-glucose (figure 3.1). The result from this experiment indicated that the sugar was transported and equilibrated rapidly within 2 minutes. The fractional filling at 50% or f= 0.5 ($t_{1/2}$), was 21.7 seconds. This contrasted with similar results obtained with 2-deoxy-D-glucose ($t_{1/2}$= 220.4 seconds). With the latter full equilibration is not attained as the 2-deoxy-D-glucose is phosphorylated once it is trapped inside the cell (figure 3.2). 2-deoxy-D-glucose is phosphorylated and metabolized by its incorporation into the glycolytic pathway. This phosphorylation prevents the equilibration of 2-deoxy-D-glucose when compared with non-metabolized 3-0-methyl-D-glucose.

The advantages of studying sugar transport into cells which are attached to an immobilised surface, preferably as a monolayer, compared to a suspension will be outlined. It was found that a study of sugar uptake was limited when the HITm2.2 and RINm5f cells were attached to a suitable surface. The detachment of RIN and HIT cells was reported to reduce the viability (Atkins unpublished communication), so initially it was seen to be an advantage to assay immobilised cells. The problems experienced with transport assays which were performed on immobilised cells, led to the development of

Figure 3.1: A time course for the uptake of 100μM 3-O-methyl-D-glucose at 20°C in the HITm2.2 cell line.
$t_{1/2}$ = 21.70 seconds

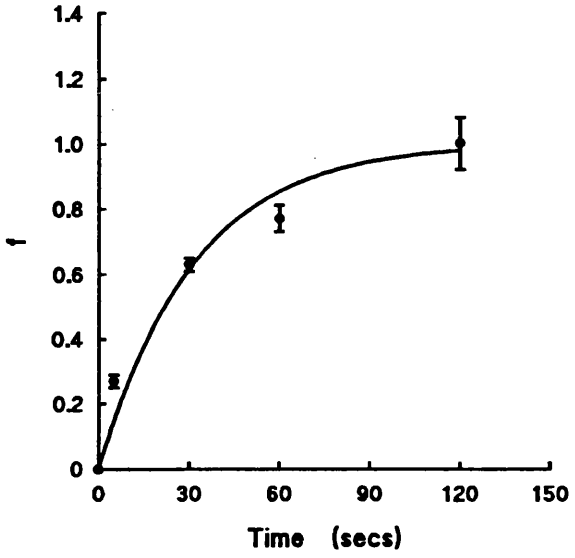
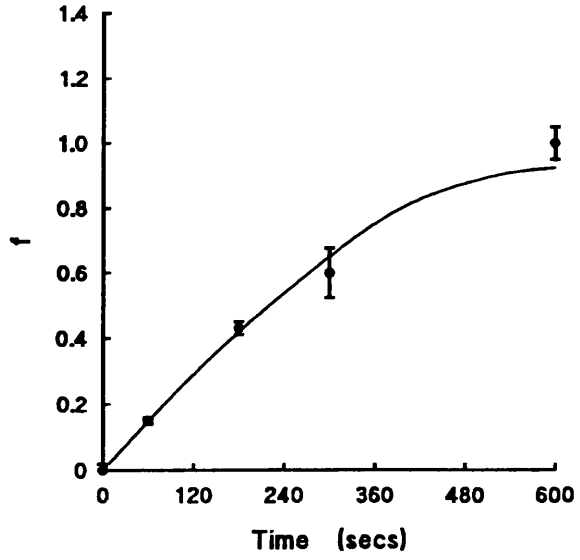


Figure 3.2: Time course for the uptake of 100μM 2-deoxy-D-glucose at 20°C in the HITm2.2 cell line.
$t_{1/2}$ = 220.4 seconds



a suspension assay. This enabled a more detailed kinetic analysis to be performed with 6-deoxy-D-glucose. The problems experienced with the immobilised cell assay, together with the adjustments made to this assay are outlined below:

1. The cells were observed to detach very easily from the culture dish upon addition of the assay buffer. This prevented the accurate determination of the cell protein between different experimental samples.

2. The final quantity of radiolabelled sugar was increased approximately ten fold. This however, did not compensate for the low CPM's obtained when the sugar attained isotope equilibrium.

3. For sugar uptake to be estimated the assay volume was decreased. From the results this volume reduction was observed to increase the CPM's obtained when the sugar attained equilibrium. This volume reduction also resulted in the cells drying out over longer incubation times.

4. Sugar transport was measured at 37^{o}C. It was difficult to maintain this temperature accurately for the duration of the experiment. As a result of the temperature fluctuations all subsequent transport experiments were performed at 22^{o}C.

A series of uptake experiments in HITm2.2 cells were performed with 2-deoxy-D-glucose and 3-O-methyl-D-glucose. From these preliminary studies,

sugar transport was shown to be inhibited by two standard sugar transport inhibitors. These were phloretin and cytochalasin B. These early experiments were performed using an immobilised cell assay. This should therefore be accounted for when interpreting the data from these experiments. Figure 3.3 illustrates the result of cytochalasin B inhibition on the transport of 3-0-methyl-D-glucose into the HIT m2.2 cell line. Sugar transport in the presence of cytochalasin B is reduced approximately 1.25 fold, when compared with a suitable control. Similarly 2-deoxy-D-glucose uptake in the presence of 100 µM phloretin (figure 3.4) is also illustrated. After a time interval of 10 minutes 2-deoxy-D-glucose uptake in the presence of phloretin is 3.5 fold lower than the appropriate control experiment. 3-0-methyl-D-glucose uptake in the presence of 100 µM phloretin (figure 3.5) is also reduced to around two fold lower than the control at an equilibrium time of 1 minute. The CPM'S s obtained with 3-0-methyl-D-glucose decreased fractionally after isotope equilibrium had been reached (between 30 and 90 seconds). This observed CPM decrease is due to a back flux of the non-metabolised label, from the inside to the outside of the cell. This contrasted with the transport of 2-deoxy-D-glucose. As this sugar is metabolized it is immediately phosphorylated once it enters the cell. The attachment of

the phosphate group to 2-deoxy-D-glucose prevents its backflux or exit from the cell.

3.1.2: The calculations for the kinetic analysis of 6-deoxy-D-glucose transport

Non-metabolised 6-deoxy-D-glucose which is not easy to obtain commercially, was used for a series of transport experiments. The initial transport studies were performed to optimise the assay. Results from preliminary time course experiments indicated that 6-deoxy-D-glucose was transported, but the assay procedure required some modifications.

Preliminary time course experiments indicated that 6-deoxy-D-glucose reached isotope equilibrium faster than metabolized 2-Deoxy-D-glucose. It was apparent that 70 volumes of the ice cold HEPES buffer, with the inclusion of phloretin at a 0.1mM final concentration, was an effective stopping agent. The following final modifications were made in adjusting the 6-deoxy-D-glucose assay:

1. A reduction in assay volume from 100 to 30 µl.
2. An increase in the cell number from 2 x 10^7 to 10x10^7 cells per assay.
3. A greater amount of labelled 6-deoxy-D-glucose was included to increase the CPM's attained at isotope equilibrium.

4. A decrease in the assay incubation times when estimating the initial rates of sugar uptake was required because of the high rate of hexose transport.

5. A reduction of the assay temperature from 37°C to 22°C

Calculations for both the fractional filling and the velocity for the uptake of 6-deoxy-D-glucose are defined in equations a and b (chapter 2). The various equilibrium values were obtained by the incubation of the cultured cells for 60 to 120 seconds with radiolabelled 6-deoxy-D-glucose. Using this value, the fractional filling f was thus calculated as from equation a. For the single time point assays used in the inhibition studies, an incubation time of 10 to 15 seconds was used. This incubation time represented a fractional filling of approximately 40% for the assays overall.

In calculating the uptake of 6-deoxy-D-glucose a simplified form of the Eliam and Stein (1974) equation was used. This equation defines the net entry of the sugar label into the cell and is illustrated in equation b. To determine the Michaelis Menten (K_m) constant and the maximal velocity (V_{max}) values, a s/v against s plot was plotted for both 6-deoxy-D-glucose and D-fructose. The Inhibition constants (K_i s) were calculated from the following equation (Holman et al., 1981):

$$\frac{v_o}{v} = 1 + \frac{I}{K_i} \qquad \text{equation c}$$

From the above equation v is the inhibited rate of sugar transport, v_o the uninhibited rate and I is the inhibitor concentration.

3.1.3: 6-deoxy-D-glucose transport and its inhibition by D-glucose analogues

For the estimation of the inhibition constants of the D-glucose analogues, the technique has tended to over estimate the final K_i. This applies to both 6-deoxy-D-glucose and the D-fructose experiments.
To establish the nature and specificity of the transporter in the HITm2.2 cell line, a series of experiments with non-metabolised 6-Deoxy-D-glucose were performed. These experiments also included both 6-deoxy-D-glucose uptake and K_m determinations. The results of a time course (Figure 3.6) for 6-deoxy-D-glucose uptake at 20^{o}C, demonstrated that this analogue was transported with a 50% fractional filling ($t_{1/2}$) of 17.3 ± 1.4 seconds. The equilibration of 6-deoxy-D-glucose was reached in 1 minute. Transport specificity was investigated by using a series of analogue inhibitors of 6-deoxy-D-glucose transport. The

Figure 3.7: A Hanes plot of the uptake of 6-deoxy-D-glucose, using a 12 second single time point assay at 20°C in the HITm2.2 cell line.
K_m= 2.1 ± 0.4mM; V_{max} = 0.21 ± 0.03 $mMsec^{-1}$

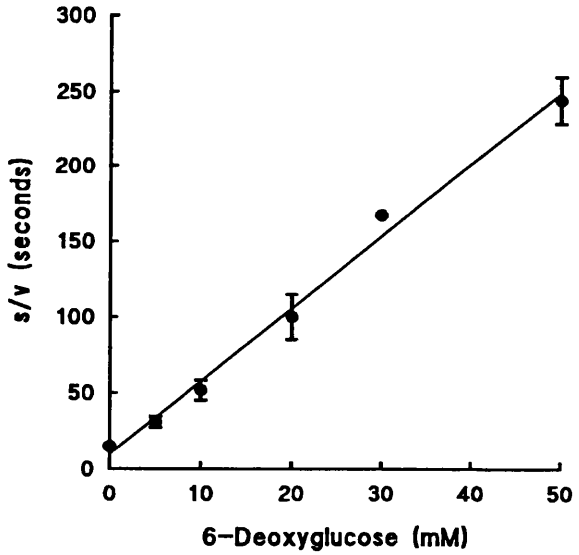


Figure 3.8: Inhibition of 100μM 6-deoxy-D-glucose uptake by D-glucose,in the HITm2.2 cell line.
K_i = 2.0 ± 0.6 mM

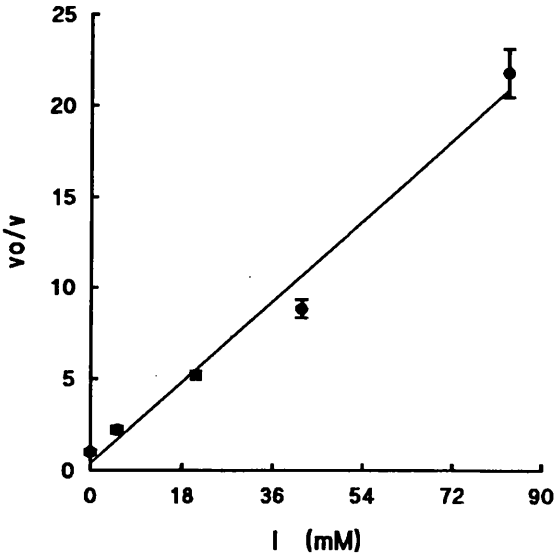


Figure 3.9: Inhibition of 100μM 6-deoxy-D-glucose uptake by D-fructose, in the HITm2.2 cell line.
K_i = 228.3 ± 84.6mM

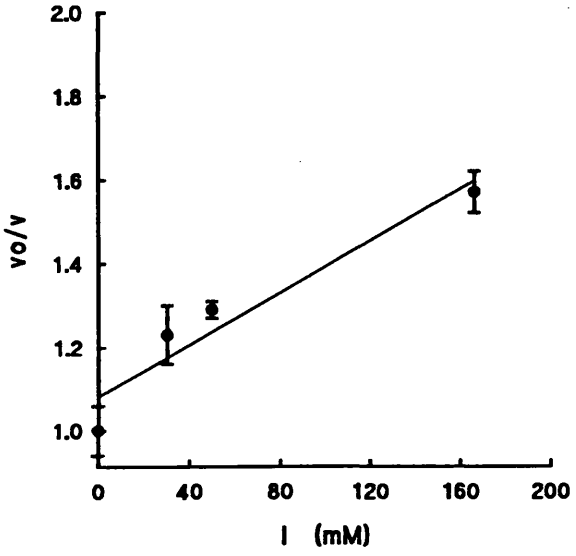
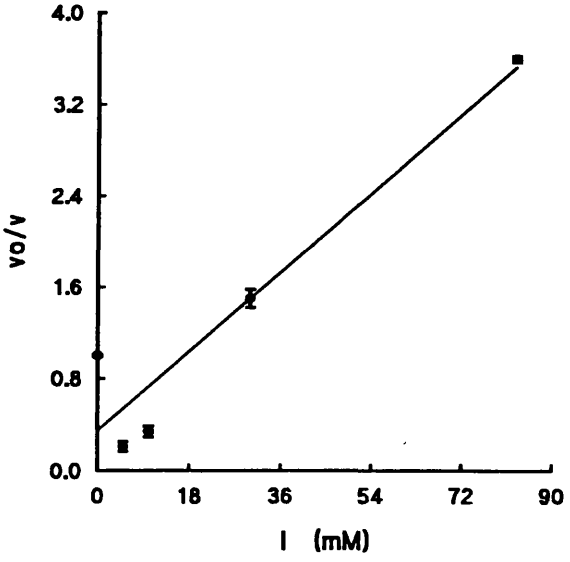


Figure 3.10: Inhibition of 100μM 6-deoxy-D-glucose uptake by D-mannose, in the HITm2.2 cell line.
K_i = 9.0 ± 2.4 mM



aim was to obtain a series of inhibition constants (K_i's) for a range of analogues at each hexose carbon position. From these experiments, both the spatial and hydrogen bonding requirements of the transporter binding site could be determined. The inhibition constants were calculated by using a range of sugar inhibitor concentrations at a fixed 100 μM 6-deoxy-D-glucose concentration. The results were subsequently plotted on a Hanes plot and fitted by linear regression.

Initially it was investigated whether preincubation with the inhibitor or the simultaneous addition of the inhibitor and 6-deoxy-D-glucose would be the most appropriate for the assay procedure. It was found to be more convenient to add the detached cells (small volume) to the inhibitor and the 6-deoxy-D-glucose tracer (large volume) for the purpose of assay accuracy and repeatability. A Hanes plot for the uptake of 6-deoxy-D-glucose is shown in figure 3.7. From these results a K_m = 2.10 ± 0.40 mM and a v_{max} = 0.21 ± 0.03 mM sec^{-1} have been determined.

The potential substrate's D-glucose, D-fructose, D-mannose and D-galactose were used as sugar inhibitors of 6-deoxy-D-glucose transport. Figures 3.8, 3.9, 3.10 and 3.11 illustrate results from which inhibition constants were calculated to be: 2.0 ± 0.6 mM for D-glucose, 228.3 ± 84.6mM for D-fructose, 9.0 ± 2.4mM for D-mannose and 21.0 ± 4.2 for D-galactose. Conclusions

Figure 3.11: Inhibition of 100μM 6-deoxy-D-glucose uptake by D-galactose, in the HITm2.2 cell line.
K_i = 21.0 ± 4.2mM

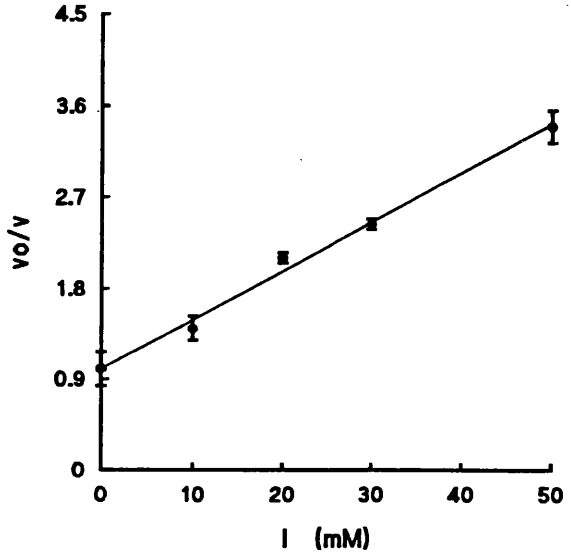
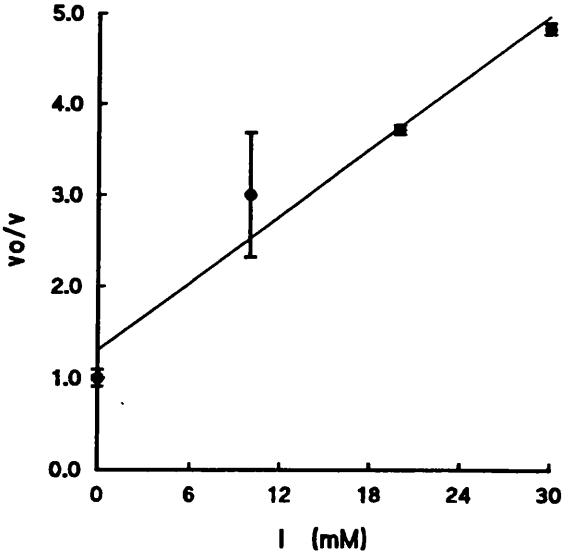


Figure 3.12: Inhibition of 100μM 6-deoxy-D-glucose uptake by 2-deoxy-D-glucose, in the HITm2.2 cell line.
K_i = 6.3 ± 1.8 mM



from these results indicate that the natural substrate D-glucose, has the expected highest affinity for the transporter binding site. D-fructose has the lowest affinity for the transporter binding site, followed by D-galactose and finally D-mannose.

Inhibition by carbon-1 analogues

There were no suitable glucose derivatives available to study the one position on the hexose ring.

Inhibition by carbon-2 analogues

The specificity study at the carbon-2 position used the available analogues, 2-deoxy-D-glucose and 2-deoxy-D-galactose as potential inhibitors of 6-deoxy-D-glucose uptake.

The K_i values obtained were 6.3 ± 1.8mM for 2-deoxy-D-glucose and 43.2 ± 17.6mM for 2-deoxy-D-galactose as shown in figures 3.12 and 3.13. The carbon-2 epimer D-mannose (figure 3.10) was found to inhibit with a K_i of 9.0 ± 2.4mM.

Inhibition by carbon-3 analogues

The carbon 3 position was analysed using the 3-O-methyl-D-glucose analogue. From figure 3.14 the inhibition constant obtained was 10.0 ±0.2 mM. The 3 positional hydroxyl group appears to demonstrate a moderate binding affinity for the transport site.

Figure 3.13: Inhibition of 100μM 6-deoxy-D-glucose uptake by 2-deoxy-D-galactose, in the HITm2.2 cell line.
K_i = 43.2 ± 17.6mM

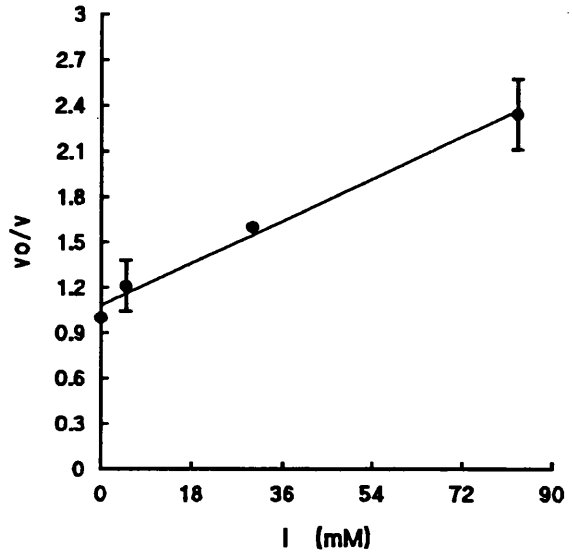
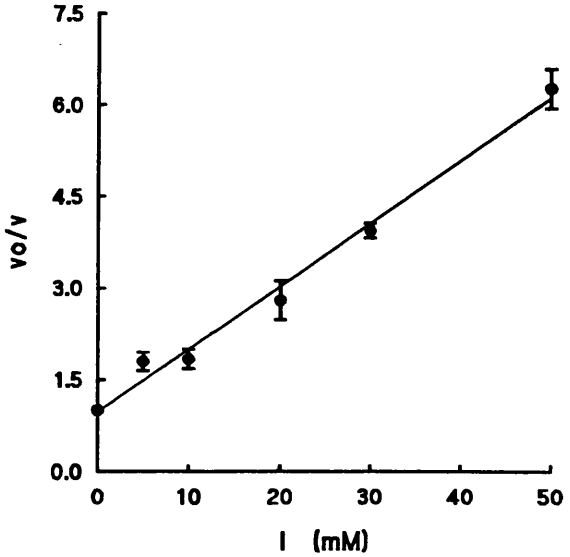


Figure 3.14: Inhibition of 100μM 6-deoxy-D-glucose uptake by 3-O-methylglucose, in the HITm2.2 cell line.
K_i = 10.0 ± 0.2 mM



Inhibition by carbon-4 analogues

As deoxy analogues were unavailable for this study, 4,6-0-ethylidene-D- glucose was used as the only inhibitor of 6-deoxy-D-glucose transport. The K_i value obtained was 100.0 ±3.8mM (figure 3.15). It can be inferred from this inhibition study that 4,6-0-ethylidene-D-glucose demonstrates a partial inhibition of 6-deoxy-D-glucose uptake.

Inhibition by carbon-5 analogues

There were no deoxy or thio glucose derivatives available to study the five positional oxygen.

Inhibition by carbon-6 analogues

The analogues used to study the six position hydroxyl were 6-deoxy-D-glucose and 6-deoxy-D-galactose. From figure 3.7 the K_m for 6-deoxy-D-glucose was calculated to be 12.0 ± 2.3 mM. 6-deoxy-D-galactose has no detectable affinity for the glucose transporter in this particular cell line. Therefore no K_i was determined. The fungal inhibitor cytochalasin B, from figure 3.16 will inhibit 6-deoxy-D-glucose transport with a K_i= 1.65 ± 0.01 μM. Therefore this compound binds with a high affinity to the transporter. Table 3.1 outlines the results of all of the inhibition studies on the uptake of 6-deoxy-D-glucose. This table represents the complete specificity analysis as allowed by the availability of analogues for use as

Figure 3.15: Inhibition of 100μM 6-deoxy-D-glucose uptake by 4,6-0-ethylidene-D-glucose, in the HITm2.2 cell line.
K_i= 100.0± 3.8 mM

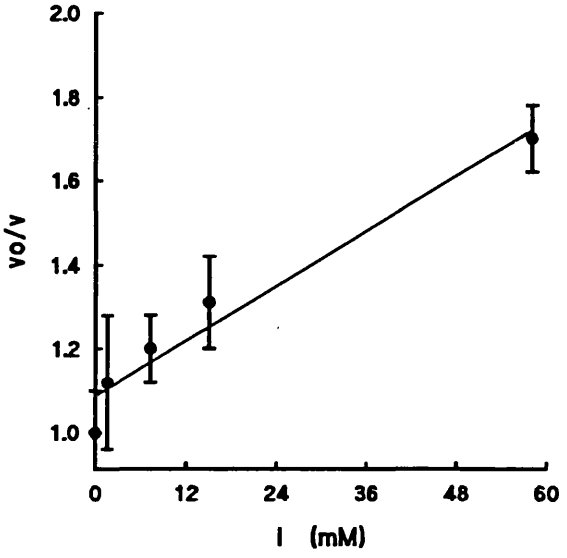
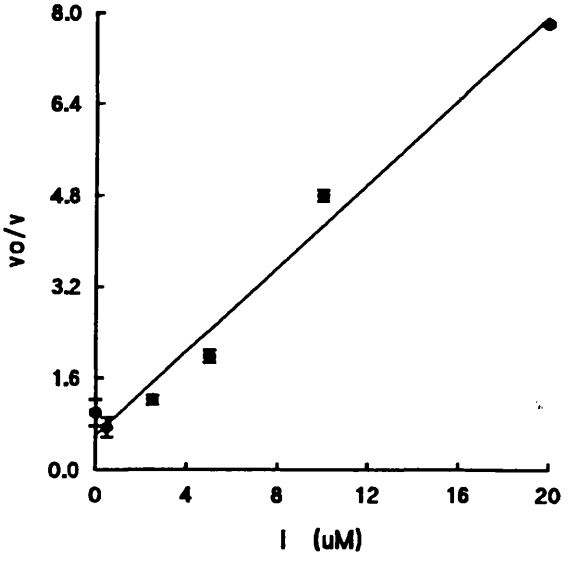


Figure 3.16: Inhibition of 100μM 6-deoxy-D-glucose uptake by the fungal inhibitor cytochalasin B, in the HITm2.2 cell line.
K_i = 1.65 μM



inhibitors. A calculation of the intracellular water space for 6-deoxy-D-glucose in the HITm2.2 cell line was performed. This calculation was determined from a time course of the uptake of 6-deoxy-D-glucose. Inferences from the experimental results indicated that the sugar uptake was fairly rapid. The 50% fractional filling was determined to be 12 seconds and an equilibrium was reached and maintained in one minute.
Using the number of CPM'S retained inside the cell at equilibrium and the specific radioactivity of the substrate, the intracellular volume accessible to the analogue was calculated to be 0.96 µl per 10^8 cells.

The use of a non transported labelled marker to determine the proportion of CPM'S in the extra cellular space of the pellet was not considered necessary. As the CPM'S in the time zero determinations were low. This was due to the large volume of the stopper solution which was used to wash the cells.

3.1.4 : D-fructose transport and its inhibition by 6-deoxy-D-glucose

Radiolabelled D-fructose experiments were performed in accordance to the 6-deoxy-D-glucose protocol. In contrast to the erythrocyte type, the islet and liver type transporters are reported to transport fructose (Gould et al., 1991). A time course for the uptake of

Figure 3.17: Time course for the uptake of 100μM D-fructose versus time at 20°C, in the HITm2.2 cell line. ($t_{1/2}$) = 15.8 ± 1.4 seconds

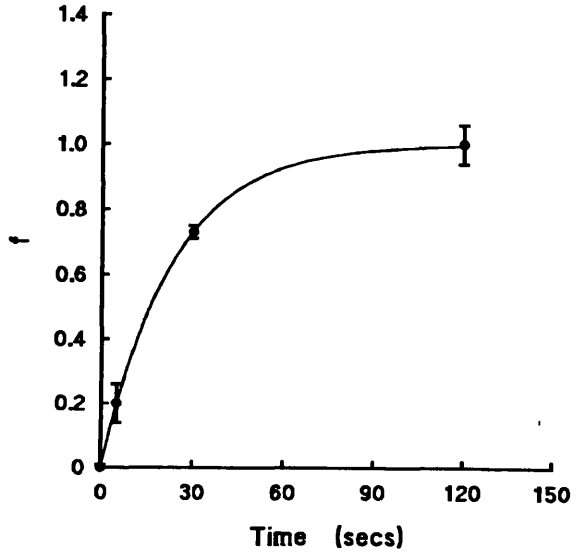
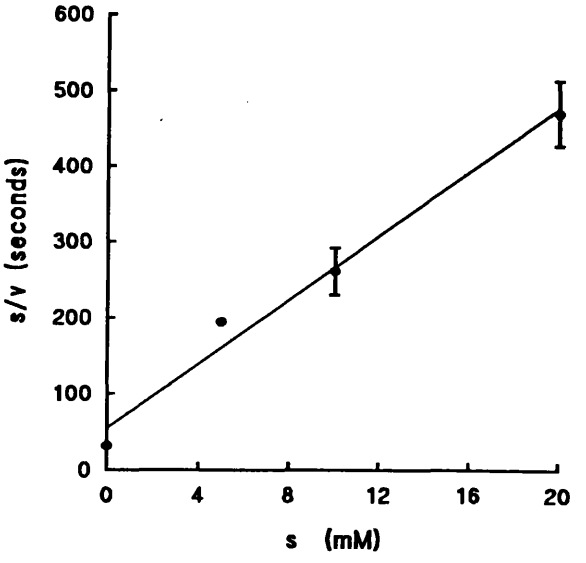


Figure 3.18: A Hanes plot of D-fructose uptake using a 12 second single time point assay at 20°C, in the HITm2.2 cell line.
K_m= 2.58± 0.2 mM; V_{max} = 0.047 ± 0.02mMs^{-1}



HITm2.2 cell line 6-deoxy-D-glucose (100 μM)	INHIBITOR	K_i (mM) (μM where stated)
Substrate	D-glucose	2.0 ± 0.6
Substrate	D-fructose	228.3 ±84.6
Substrate	D-mannose	9.0 ± 2.4
Substrate	D-galactose	21.0 ± 4.2
C-2 Analogue	2-deoxy-D -glucose	6.3 ± 1.8
	2-deoxy-D -galactose	43.2 ± 17.6
C-3 Analogue	3-0-methyl-D -glucose	10.0 ± 0.2
C-4 Analogue	4,6-0-ethylidene -D-glucose	100.0 ± 3.8
C-6 Analogue	6-deoxy-D -galactose	N.D.I.
Fungal inhibitor	Cytochalasin B	1.65± 0.01 μM

Table 3.1: A table illustrating the results of an inhibition study which was performed on the HITm2.2 cell line. A range of D-glucose analogues were used to determine their ability to compete with 6-deoxy-D-glucose. N.D.I. = No Detectable Inhibition.

D-fructose was performed. Figure 3.17 shows a graphical plot from which a 50% fractional filling ($t_{1/2}$) of 15.8 ± 1.4 seconds was calculated. The equilibrium was reached and maintained in around 60 seconds. Kinetic data for D-fructose transport was calculated from figure 3.18. This data included a Km = 2.58 ± 0.20mM and a Vmax = 0.047 ± 0.02mMs^{-1} for D-fructose.

From inhibition studies in the HITm2.2 cell line, D-fructose is known to inhibit the uptake of the 6-deoxy-D-glucose analogue weakly. This K_i was determined to be 228.3 ± 84.6 mM (figure 3.9). Conversely, 6-deoxy-D-glucose will also inhibit the uptake of 100μM D-fructose (figure 3.19) with a calculated K_i of 52.0 mM. From these results it can be inferred that both of these labels are taken up by the same transporter type.

3.2 Sugar transport into the RINm5f cell line

3.2.1: 6-deoxy-D-glucose transport and 3-O-methyl-D-glucose uptake in the presence of phloretin

The uptake of 3-O-methyl-D-glucose into the RINm5f cell line in the presence of 100μM phloretin is shown in figure 3.20. Here transport under phloretin is reduced to around 1.5 fold at equilibrium.

Figure 3.19: The inhibition of 100μM D-fructose uptake by the 6-deoxy-D-glucose analogue, in the HITm2.2 cell line. K_i = 52.0 mM.

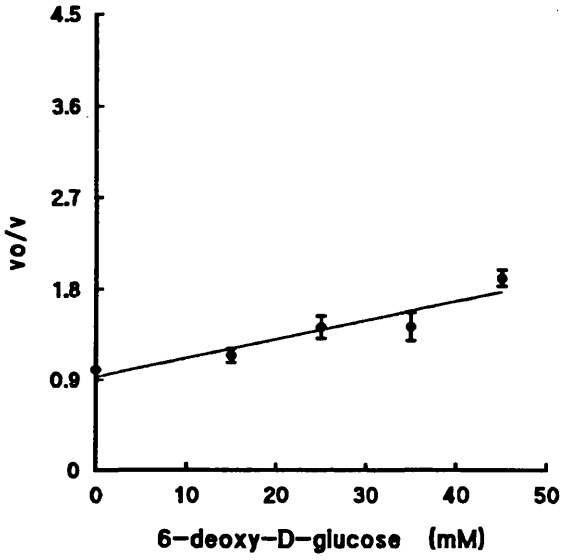
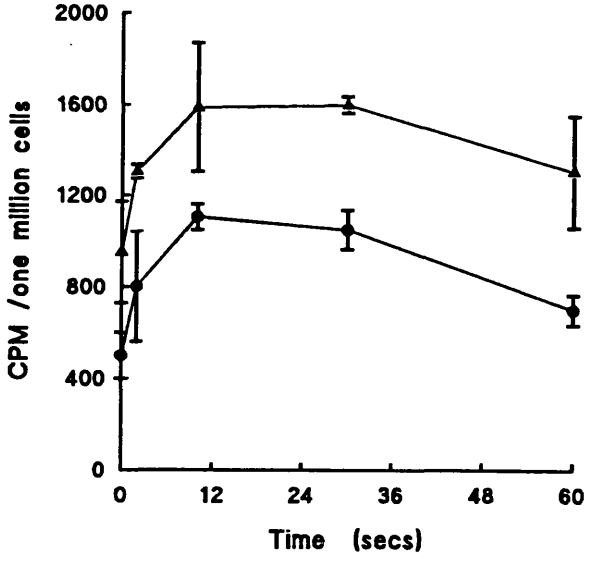


Figure 3.20: The inhibition of the uptake of 50μM 3-0-methylglucose (black triangles) in the RINm5f cell line. 3-0-methyl-D-glucose transport is inhibited by 100μM phloretin (black circles).



The RINm5f cell line was analysed the same way as the HITm2.2 cell line. A time course for the uptake of 6-deoxy-D-glucose was calculated and plotted (figure 3.21). From figure 3.21 a $t_{1/2}$ of 10 ± 0.7 seconds was determined. The sugar was equilibrated after about 90 seconds and maintained for up to 3 minutes. Figure 3.22 illustrates results of a Hanes plot for 6-deoxy-D-glucose. The K_m and V_{max} values were calculated to be 3.62 ± 0.98 mM and 0.43 ± 0.09 mMs^{-1}. The assay was developed as for the HITm2.2 cell line and a series of specificity studies were performed with various analogue inhibitors. From these studies inhibition constants (Ki's) were calculated.

3.2.2: The inhibition of 6-deoxy-D-glucose transport by D-glucose analogues

D-glucose, D-mannose, D-fructose and D-galactose were used as possible substrate's which compete with the transport of 6-deoxy-D-glucose. Figures 3.23, 3.24 and 3.25 illustrates plots from which K_i's were determined. These K_i's were calculated to be : 5.9 ± 1.0 mM for D-glucose, 32.0 ± 19.4mM for D-mannose and 51.5 ± 3.2 mM for D-galactose. D-fructose failed to inhibit 6-deoxy-D-glucose uptake with any measurable K_i. It can be inferred from these results that D-glucose (the natural substrate) has the highest affinity

Figure 3.21: A Time course for the uptake of 100μM 6-deoxy-D-glucose versus time at 20°C, in the RINm5f cell line.
$t_{1/2}$ = 10 ± 0.7 seconds

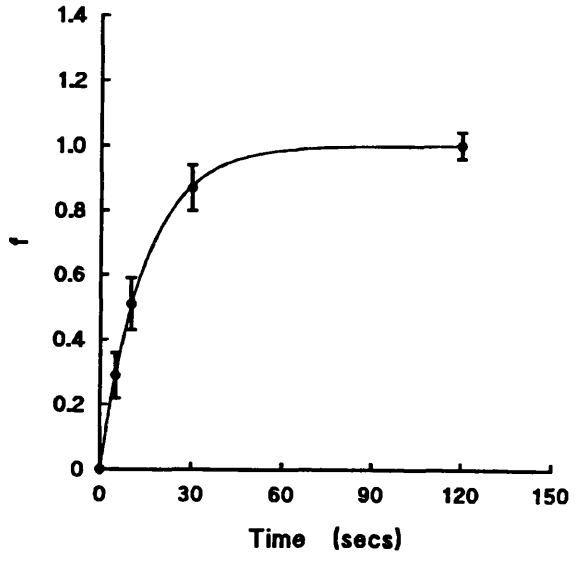


Figure 3.22: A Hanes plot of 6-deoxy-D-glucose uptake using a 15 second single time point assay at 20°C, in the RINm5f cell line.
K_m = 3.62 ± 0.98 mM; V_{max} = 0.43 ± 0.09mMs^{-1}

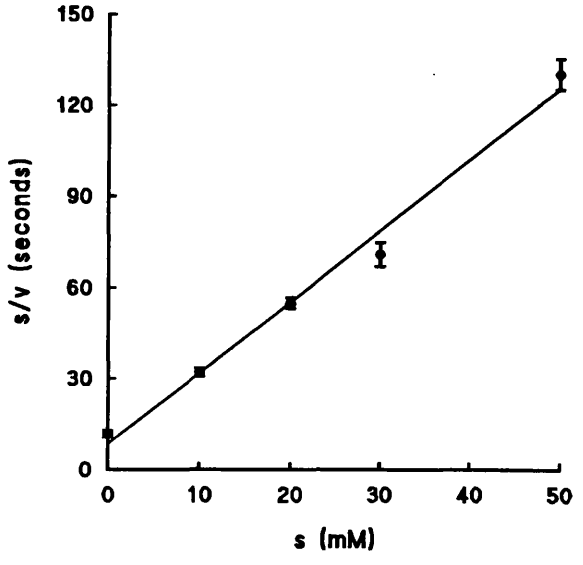


Figure 3.23: Inhibition of 100μM 6-deoxy-D-glucose uptake by D-glucose, in the RINm5f cell line.
K_i = 5.9 ± 1.0 mM

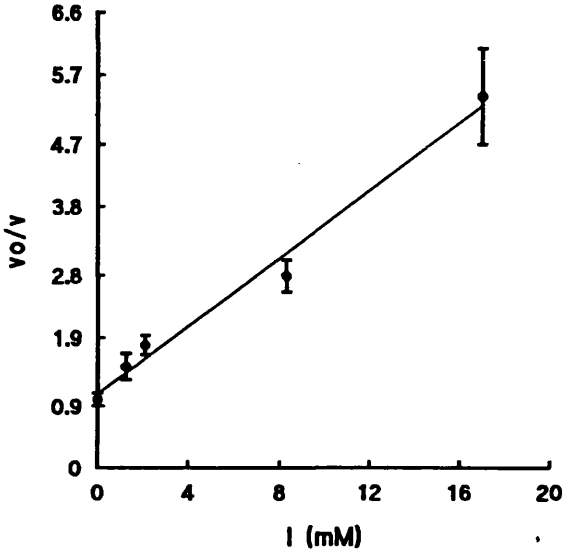
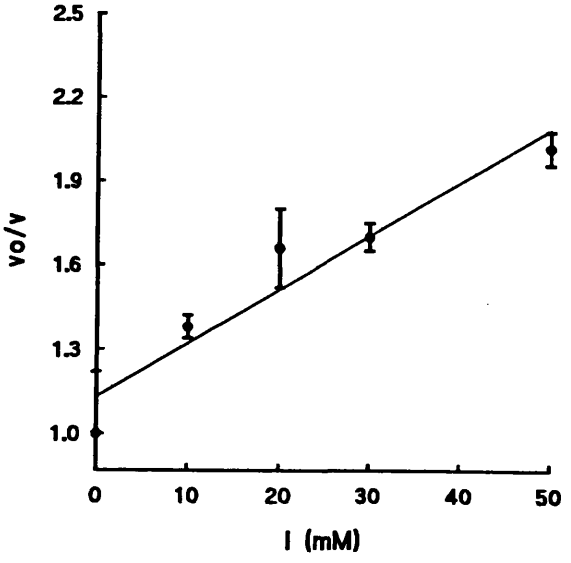


Figure 3.24: Inhibition of 100μM 6-deoxy-D-glucose uptake by D-mannose, in the RINm5f cell line.
K_i= 32.0 ± 19.4 mM



for the transporter binding site, followed by D-mannose and D-galactose. D-fructose has no ability to inhibit sugar uptake at its concentration range.

Inhibition by carbon-1 analogues

There were no analogues available to study the positional hydrogen bonding at carbon-1.

Inhibition by carbon-2 analogues

The analogues used here were 2-deoxy-D-glucose and 2-deoxy-D-galactose as inhibitors of 6-deoxy-D-glucose uptake. The Ki values obtained were 22.9 ± 4.4mM for 2-deoxy-D-glucose and 36.0 ± 0.8 mM for 2-deoxy-D-galactose from figures 3.26 and 3.27. The carbon-2 epimer D-mannose inhibited with a Ki of 32.0 ± 19.4 mM (figure 3.24).

Inhibition by carbon-3 analogues

The 3 position on the hexose ring was studied using 3-0-methyl-D-glucose. It was inferred that 3-0-methyl-D-glucose demonstrates a moderate affinity of binding to the transporter with a Ki = 9.5 ± 3.6 mM (figure 3.28).

Figure 3.25: Inhibition of 100μM 6-deoxy-D-glucose uptake by D-galactose, in the RINm5f cell line.
K_i= 51.5 ± 3.2mM

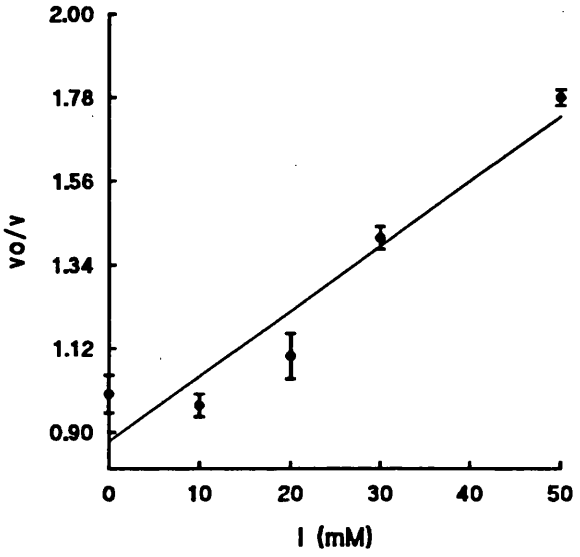


Figure 3.26: Inhibition of 100μM 6-deoxy-D-glucose uptake by 2-deoxy-D-glucose, in the RINm5f cell line.
K_i= 22.9 ± 4.4mM

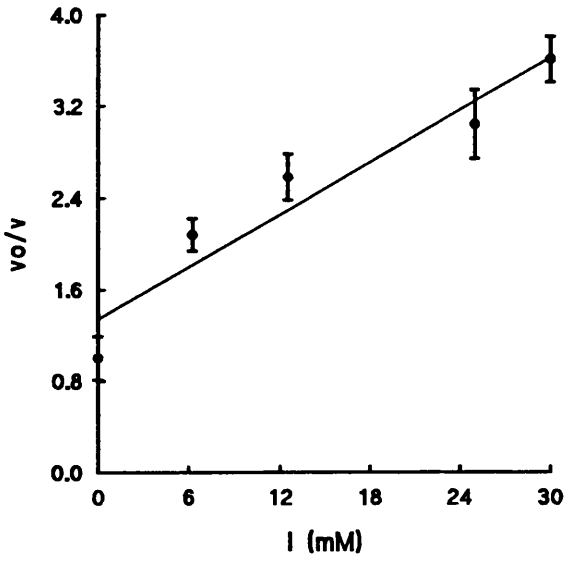


Figure 3.27: Inhibition of 100μM 6-deoxy-D-glucose uptake by 2-deoxy-D-galactose, in the RINm5f cell line.
K_i= 36.0 ±0.8 mM

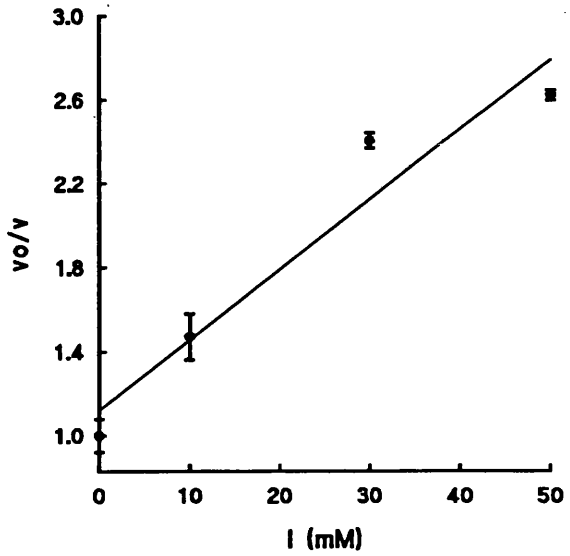
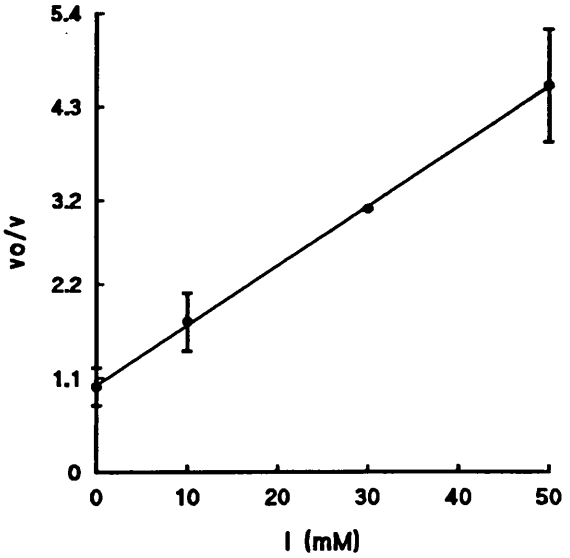


Figure 3.28: Inhibition of 100μM 6-deoxy-D-glucose uptake by 3-0-methylglucose, in the RINm5f cell line.
K_i= 9.5± 3.6mM



Inhibition by carbon-4 analogues

As deoxy analogues were not available, analysis was restricted to the 4,6-0-ethylidene-D-glucose analogue. The Ki value obtained for 4,6-0-ethylidene-D-glucose was 2.4 ± 0.6 mM (figure 3.29). This analogue has a high affinity for binding to the transporter site.

Inhibition by carbon-5 analogues

There were no deoxy or thio glucose derivatives available to study the five position or the ring oxygen.

Inhibition by carbon-6 analogues

The analogues used here were 6-deoxy-D-glucose and 6-deoxy-D-galactose. 6-deoxy-D-glucose was used as a substrate. The K_i for 6-deoxy-D-galactose was 98.4 ± 4.9 mM (figure 3.30). This analogue exhibits a weak affinity for the transporter binding site.

The fungal inhibitor cytochalasin B binds with a K_i = 9.4 ±0.4 μM (figure 3.31). From this it can be inferred that cytochalasin B binds to the transporter with a moderate affinity. Table 3.2 indicates the complete specificity analysis determined by the D-glucose analogues available for the study.

Figure 3.29: Inhibition of 100μM 6-deoxy-D-glucose uptake by 4,6-O-ethylidene-D-glucose, in the RINm5f cell line. K_i= 2.4 ± 0.6 mM

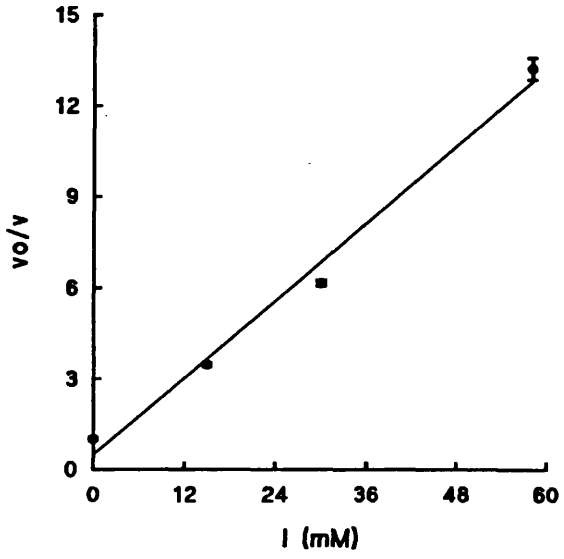


Figure 3.30: Inhibition of 100μM 6-deoxy-D-glucose uptake by 6-deoxy-D-galactose, in the RINm5f cell line. K_i= 98.4 ± 4.9mM

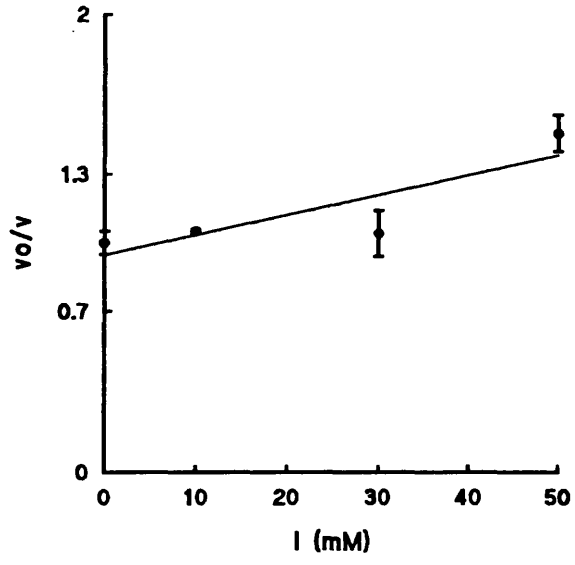


Figure 3.31: Inhibition of 100μM 6-deoxy-D-glucose uptake by cytochalasin B, in the RINm5f cell line.
K_i= 9.4 ± 0.4 μM

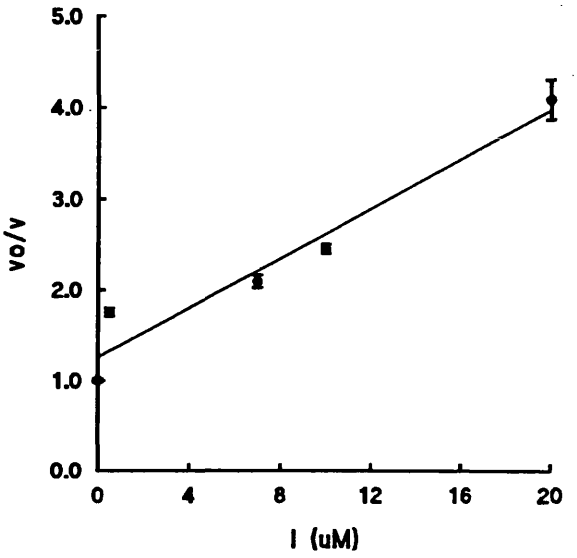
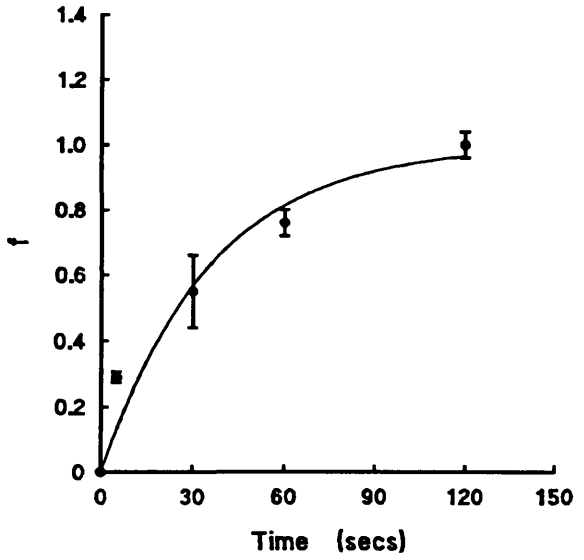


Figure 3.32: Time course for the uptake of 100μM D-fructose versus time at 20°C, in the RINm5f cell line.
$t_{1/2}$= 24.76 ± 3.60 seconds



RINm5f cell line 6-deoxy-D-glucose (100 μM)	INHIBITOR	K_i (mM) (μM where stated)
Substrate	D-glucose	5.9 ± 1.0
Substrate	D-fructose	N.D.I.
Substrate	D-mannose	32.0 ± 19.4
Substrate	D-galactose	51.5 ± 3.2
C-2 Analogue	2-deoxy-D -glucose	22.9 ± 4.4
	2-deoxy-D -galactose	36.0 ± 0.8
C-3 Analogue	3-0-methyl-D -glucose	9.5 ± 3.6
C-4 Analogue	4,6-0-ethylidene -D-glucose	2.4 ± 0.6
C-6 Analogue	6-deoxy-D -galactose	98.4 ± 4.9
Fungal inhibitor	Cytochalasin B	9.4 ± 0.01μM

Table 3.2: A table illustrating the results of an inhibition study which was performed on the RINm5f cell line. A range of D-glucose analogues were used to determine their ability to compete with 6-deoxy-D-glucose transport. N.D.I. = No Detectable Inhibition.

3.2.3: D-fructose transport and its inhibition by 6-deoxy-D-glucose

The labelled D-fructose uptake experiments were performed using the same assay conditions as for 6-deoxy-D-glucose. From figure 3.32, a time course for the uptake of D-fructose is plotted. A $t_{1/2}$ of 24.76 ± 3.60 seconds was calculated. The equilibration of D-fructose was achieved in around 2 minutes and maintained for three minutes. This represents a fairly rapid uptake of the sugar. A Hanes plot of D-fructose uptake is plotted in figure 3.33. Kinetic data was calculated and a K_m = 19.50 ± 2.50mM and V_{max} = 2.30 ± 0.36 mMs^{-1} were determined.

A parallel study with D-fructose inhibition upon the uptake of 6-deoxy-D-glucose and vice versa were performed. There is no detectable inhibition by D-fructose on the uptake of 6-deoxy-D-glucose. The results from the converse experiment also indicated no detectable inhibition. As both of the sugar labels are known to be transported into the RINm5f cell line one can infer that there is a difference in the transporter specificity, when compared to the HITm2.2 transporter. There also may be a heterogeneous transporter population present in this cell line with a transporter specific for D-fructose.

Figure 3.33: A Hanes plot of D-fructose uptake using a 15 second single time point assay at 20°C, in the RINm5f cell line.
K_m = 19.50 ± 2.50 mM; Vmax = 2.30 ± 0.36 mMs^{-1}

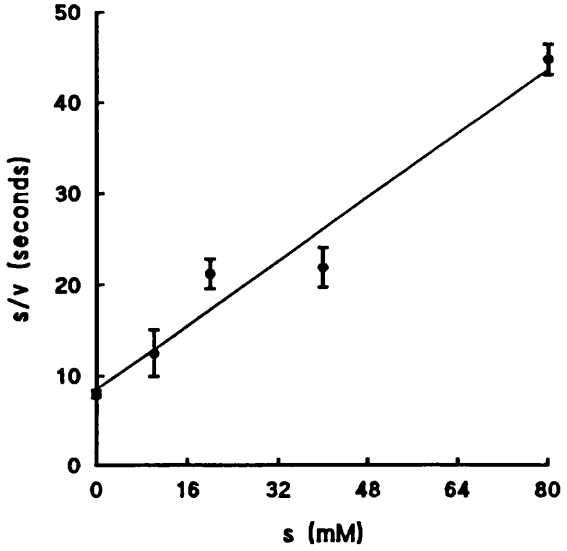
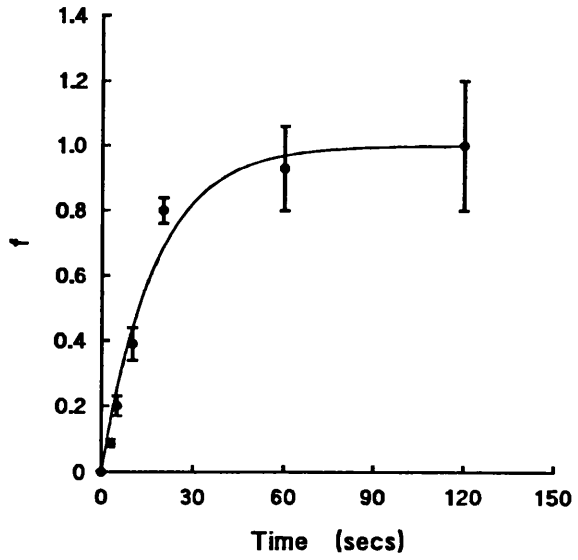


Figure 3.34: Time course for the uptake of 100µM 6-deoxy-D-glucose versus time at 20°C, in the HIT-T15 cell line.
$t_{1/2}$= 12.00± 0.35 seconds



3.3: Sugar transport into the HIT-T15 cell

3.3.1: 6-deoxy-D-glucose and D-fructose transport

A limited characterisation with 6-deoxy-D-glucose and D-fructose was performed with this cell line. Initially these cells were difficult to grow under tissue culture conditions. The growth conditions of HIT-T15 cells were finally optimised to obtain the quantity of cells which were required for transport assays to be performed. There was a slight modification in the sugar uptake assay for both HIT-T15 and MIN-6 cell. Here the assay was performed with the cells attached to the tissue culture dishes. A time course for the uptake of 6-deoxy-D-glucose is plotted in figure 3.34. From this a $t_{1/2}$ of 12.0 ± 0.4 seconds was determined. 6-deoxy-D-glucose was equilibrated in around 2 minutes. A Hanes plot of 6-deoxy-D-glucose transport data is plotted in figure 3.35. From this graph the following kinetic data were determined. This included a K_m = 8.33 ±0.83 mM and a V_{max} = 0.32 ±0.20 mMs^{-1} for 6-deoxy-D-glucose. From a plot of the fractional filling for D-fructose uptake (figure 3.36), a $t_{1/2}$ of 23.2 seconds was determined. D-fructose reached its equilibrium in 2 minutes. Further kinetic analysis revealed a K_m of 0.26 ± 0.01 mM with a V_{max} = 0.37 ± 0.11 mMs^{-1} for D-fructose (figure 3.37). The intracellular water space accessible to

Figure 3.35: Hanes plot of 6-deoxy-D-glucose uptake using a 15 second single time point assay at 20°C, in the HIT-T15 cell line.
K_m= 8.33 ± 0.83 mM; V_{max} = 0.32 ± 0.20mMs^{-1}

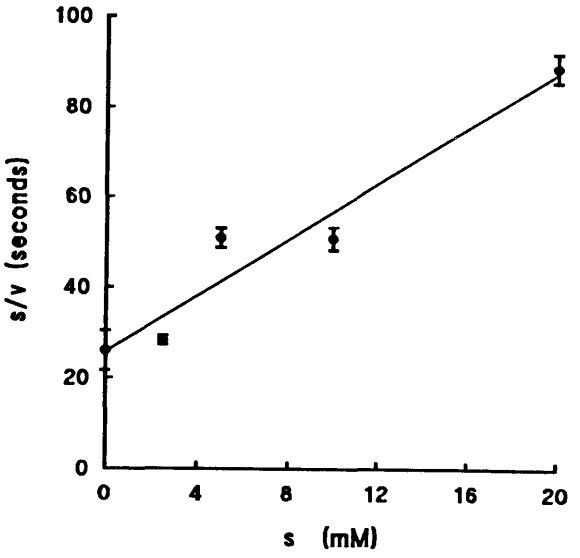
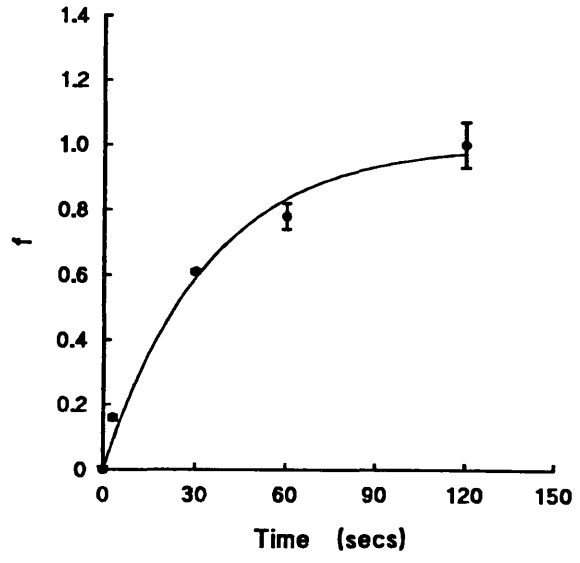


Figure 3.36: Time course for the uptake of 100μM D-fructose versus time at 20°C, in the HIT-T15 cell line.
$t_{1/2}$ = 23.20 seconds



6-deoxy-D-glucose was determined to be 0.96µl per 10^8 cells.

Further experiments looking at the inhibition of D-fructose upon the uptake of 100µM 6-deoxy-D-glucose and vice versa, indicated no detectable inhibition. It is likely that these sugars are transported via separate transport proteins.

3.3.2: D-streptozotocin transport and its inhibition by 6-deoxy-D-glucose and D-fructose

The uptake of 100 µM streptozotocin into HIT-T15 cells was performed using essentially the same experimental transport method as for D-fructose and 6-deoxy-D-glucose. Figure 3.38 illustrated a graphical plot depicting the uptake of 100µM streptozotocin into the HIT-T15 cell line. From this plot the $t_{1/2}$ was calculated to be 57.8 seconds. D-streptozotocin was equilibrated in 3 to 4 minutes.

A series of inhibition studies were performed on the uptake of streptozotocin in this cell line. D-fructose and 6-deoxy-D-glucose were used as substrate's for these inhibition studies. Figure 3.39, illustrates a graphical plot of 6-deoxy-D-glucose inhibition of streptozotocin transport into HIT-T15 cells. A K_i of 28.4 mM was calculated. There is no detectable inhibition of

Figure 3.37: Hanes plot of D-fructose uptake using a 15 second single time point assay at 20°C in the HIT-T15 cell line.
K_m = 0.26 ± 0.01 mM; V_{max} = 0.37 ± 0.11mMs^{-1}

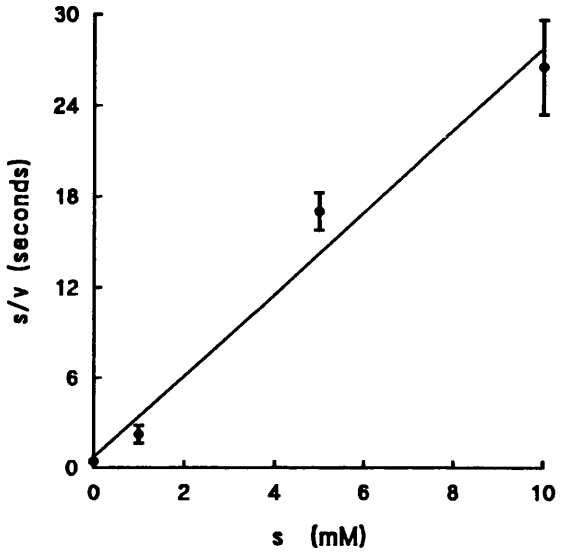


Figure 3.38: A time course for the uptake of 100µM streptozotocin versus time at 20°C in the HIT-T15 cell line.
$t_{1/2}$= 57.76 seconds.

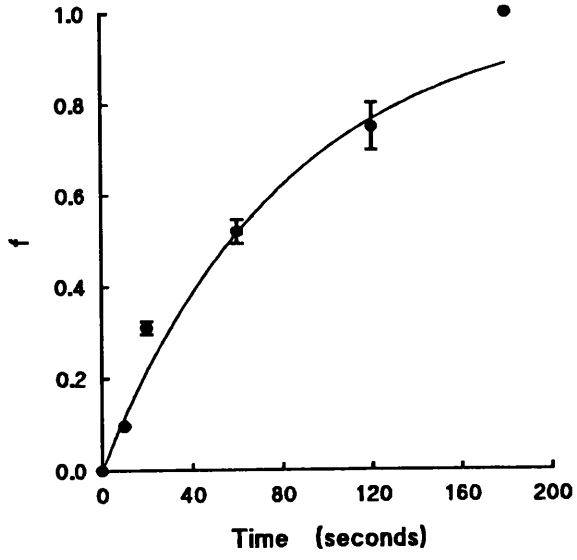


Figure 3.39: The inhibition of 100μM streptozotocin uptake by the 6-deoxy-D-glucose analogue in the HIT-T15 cell line.
K_i = 28.4 mM.

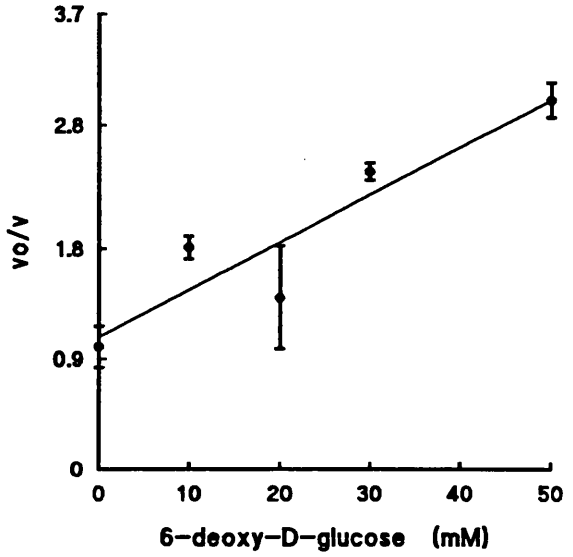
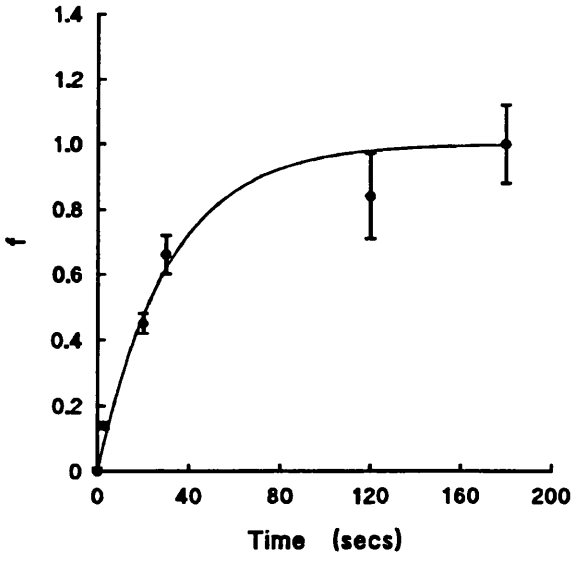


Figure 3.40: Time course for the uptake of 100μM 6-deoxy-D-glucose versus time at 20°C in the MIN-6 cell line.
$t_{1/2}$ = 21.66 ± 3.53 seconds



streptozotocin transport by D-fructose in the HIT-T15 cell line.

3.4: Sugar transport into MIN-6 cells

3.4.1: 6-deoxy-D-glucose and D-fructose transport

An analysis of 6-deoxy-D-glucose and D-fructose transport were performed with MIN-6 cells. A time course of 6-deoxy-D-glucose transport is plotted in figure 3.40. From this graph, a $t_{1/2}$ of 21.6 ± 3.5 seconds was determined and this sugar was equilibrated in around 3 minutes. Experimental results from a Hanes plot revealed the following kinetic values for 6-deoxy-D-glucose transport. A Km = 4.24± 0.44 mM and a Vmax = 0.13 ± 0.03 mMs^{-1} were determined (figure 3.41). From figure 3.42 which illustrates plot defining a time course for D-fructose transport, a $t_{1/2}$ of 35.0 ± 1.8 seconds was calculated. D-fructose attained its equilibrium in around 3 minutes. Parameters of a kinetic analysis defining D-fructose transport were calculated from figure 3.43. From this plot a K_m = 7.33 ± 0.47 mM and a V_{max} = 0.017 ± 0.009 mMs^{-1} were determined. A calculation of the intracellular water space accessible to 6-deoxy-D-glucose in this cell line was carried out. From this calculation a value of 0.63 µl per 10^8 cells was determined.

Figure 3.41: Hanes plot of 6-deoxy-D-glucose uptake using a 15 second time point assay at 20°C in the MIN-6 cell line.
K_m = 4.42 ± 0.44 mM; V_{max} = 0.13 ±0.03 mMs^{-1}

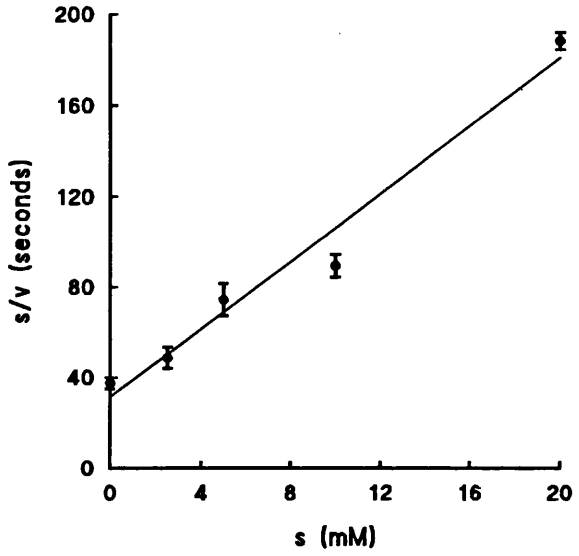
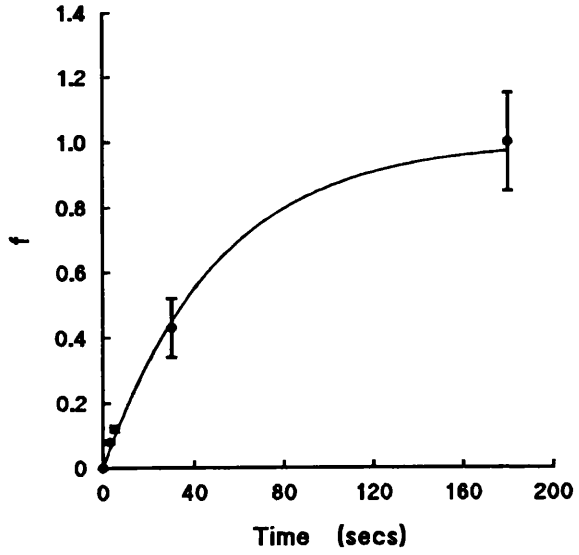


Figure 3.42: Time course for the uptake of 100μM fructose versus time at 20°C in the MIN-6 cell line.
$t_{1/2}$= 35.0± 1.8 seconds



From figure 3.44 in the MIN-6 cell line, D-fructose is reported to inhibit the uptake of 100μM 6-deoxy-D-glucose. A K_i = 60.5 mM was calculated. Conversely the K_i for the inhibition of 100 μM D-fructose (figure 3.45) uptake by the 6-deoxy-D-glucose was calculated to be 23.5 mM. From this it can be deduced that 6-deoxy-D-glucose and D-fructose are transported by the same transporter.

3.4.2: D-streptozotocin transport and its inhibition by 6-deoxy-D-glucose and D-fructose

The plot of the fractional filling defining the uptake of 100μM streptozotocin into the MIN-6 cell line is illustrated in figure 3.46. From this figure an equilibrium is attained in 3 minutes. A $t_{1/2}$ for streptozotocin of 30.8 seconds was also determined. In the MIN-6 line, 6-deoxy-D-glucose inhibits streptozotocin uptake with a Ki = 14.0 mM (figure 3.47). From figure 3.47 6-deoxy-D-glucose inhibits transport with a greater affinity than in HIT-T15 cells (figure 3.39). Figure 3.48 demonstrates a plot for the inhibition of D-fructose on the transport of 100μM streptozotocin. The sugar analogue inhibits with a calculated K_i of 33.3 mM.

Figure 3.43: A Hanes plot of D-fructose uptake using a 15 second single time point assay at 20°C in the MIN-6 cell line.
K_m = 7.33 ± 0.47 mM; V_{max} = 0.02 ± 0.009 mMs^{-1}

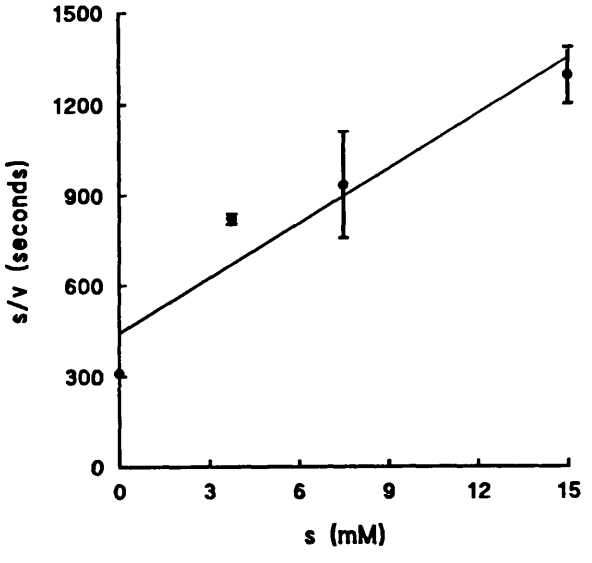


Figure 3.44: The inhibition of 100μM 6-deoxy-D-glucose uptake by the D-fructose analogue in the MIN-6 cell line.
K_i = 60.5 mM.

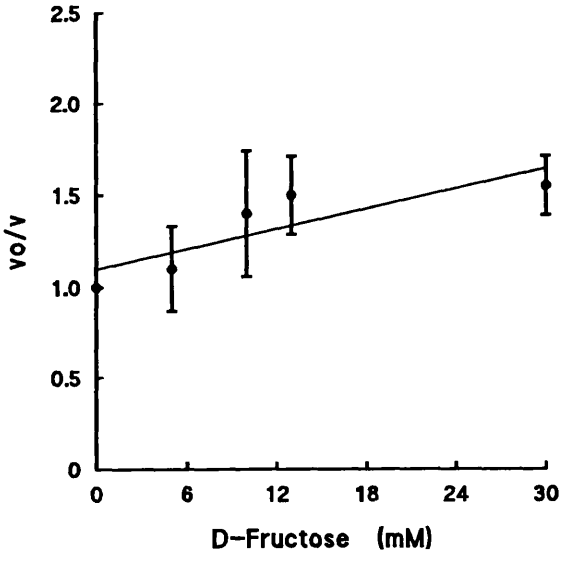


Figure 3.45: The inhibition of 100μM D-fructose uptake by the 6-deoxy-D-glucose analogue in the MIN-6 cell line.
K_i = 23.5 mM.

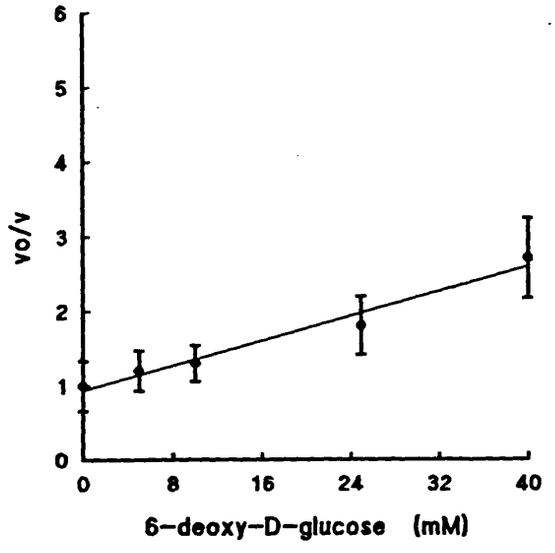
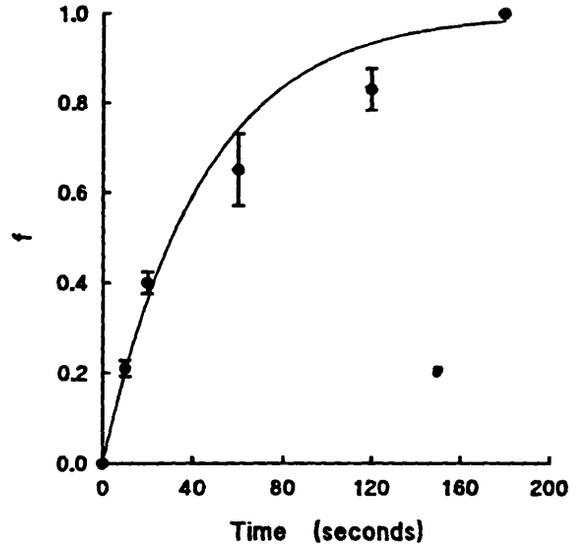


Figure 3.46: A time course for the uptake of 100μM streptozotocin versus time at 20°C in the MIN-6 cell line.
$t_{1/2}$= 30.80 seconds.



3.5: An investigation of streptozotocin binding to the erythrocyte transporter

It has been established that there are a number of multiple binding sites for cytochalasin B on the erythrocyte transporter. The use of cytochalasin E has enabled cytochalasin B (a classical fungal inhibitor of sugar transport), in the presence of cytochalasin E, to bind specifically to a single high affinity site on the erythrocyte transporter (Baldwin et al., 1982). Cytochalasin E is included to bind to the non-specific sites which would otherwise be occupied by the ligand of interest cytochalasin B.

To be able to study both the binding and the transport of the diabetogenic drug streptozotocin, it was decided to use erythrocyte membranes as these were readily available. In addition the erythrocyte transporter is also a well defined system to use as a preliminary model. Such membranes were used to look at the interaction of the drug with binding sites on the membranes. It was anticipated that streptozotocin would compete strongly with cytochalasin B for a mutual binding site. The analysis took the form of some simple kinetic treatment. This treatment included the inhibition of cytochalasin B by streptozotocin being studied by two approaches. Such a study would enable the precise

interaction of the drug to be determined in a well defined model system.

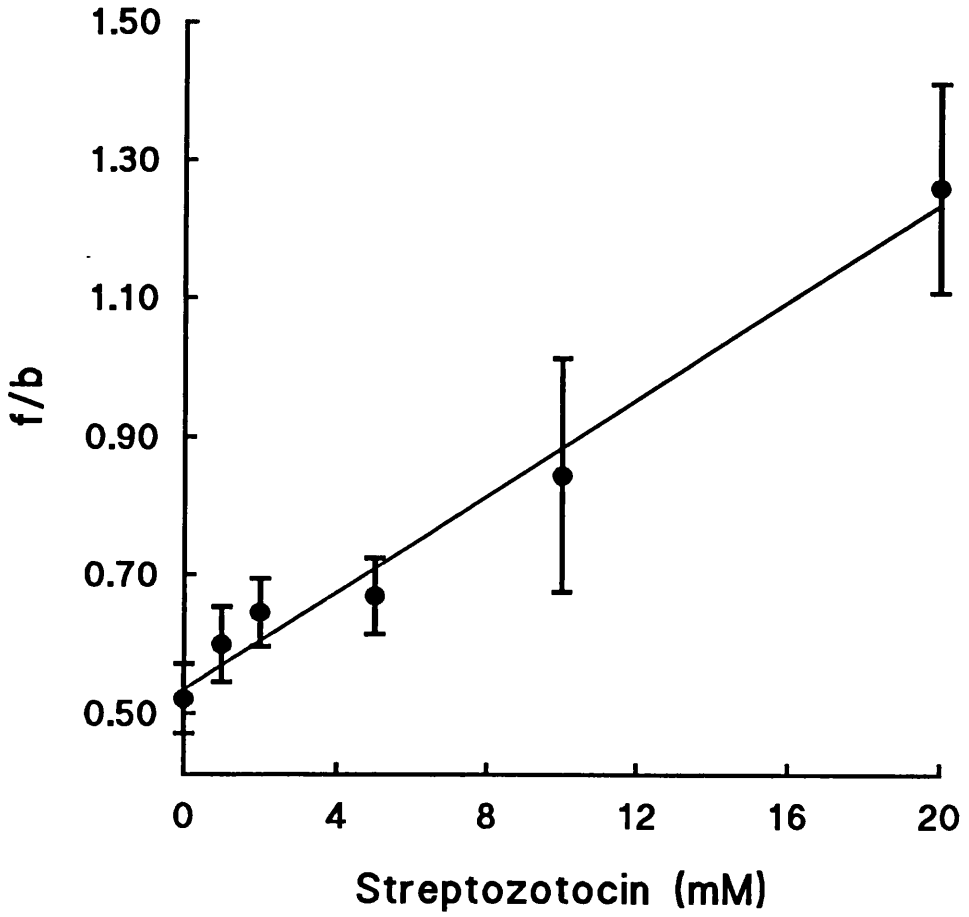
3.5.1: Cytochalasin B binding in the presence of streptozotocin

This assay was performed with a suitable range of streptozotocin concentrations (0 to 20 mM). The inhibition of the binding of radiolabelled cytochalasin B was then studied. In the following study, both the inhibitor and the substrate were added to the membrane preparation simultaneously to minimise any streptozotocin degradation that may occur in solution. Figure 3.49 illustrates a plot which defines the inhibition by streptozotocin on the binding of cytochalasin B to the membrane.

Results from this experiment were then subjected to a modified Scatchard treatment upon the assumption that cytochalasin B will bind to a single high affinity site. The Scatchard equation can be written in the following form shown below:

$$[B] = \frac{[B_{max}] \cdot [F]}{K_d + [F]}$$

Figure 3.49 : A plot illustrating the inhibition of cytochalasin B binding to the erythrocyte membrane. The inhibitor is streptozotocin. Free/ bound cytochalasin B is plotted against the concentration of streptozotocin. The inhibition constant (K_i) for streptozotocin is calculated to be 1.89 mM



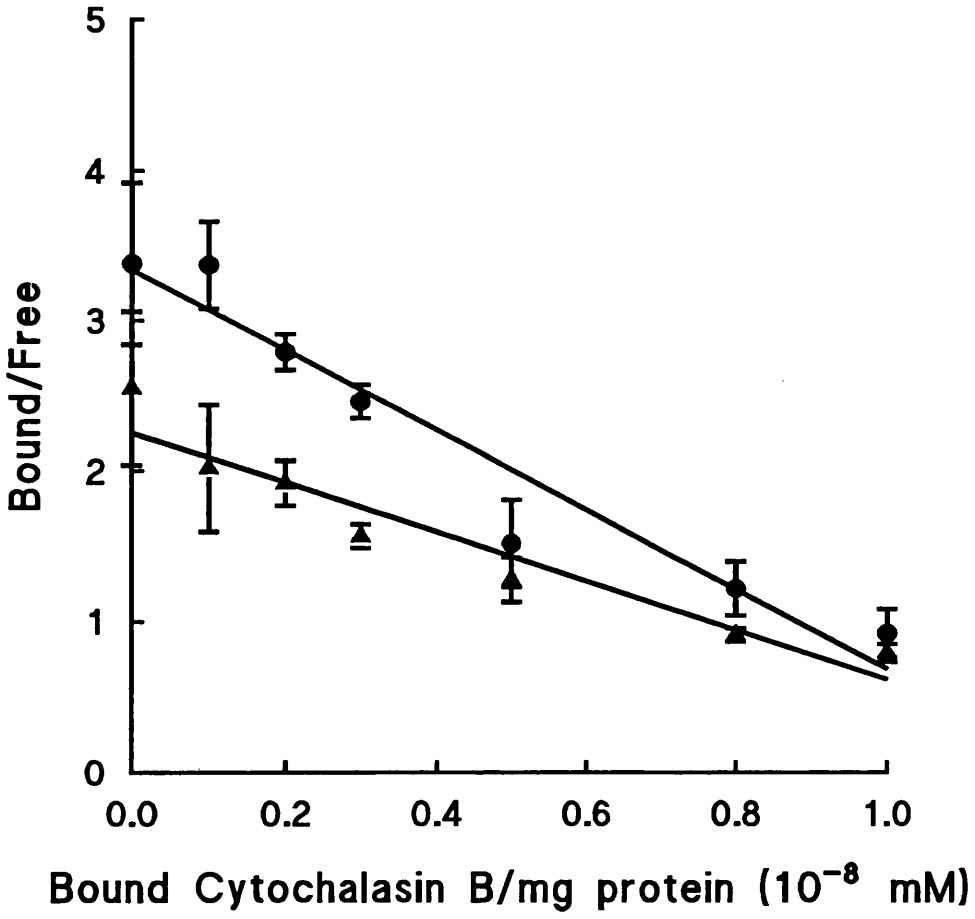
From this equation [B] is the concentration of the bound ligand, [F] is the free ligand concentration, K_d is the dissociation constant and $[B_{max}]$ represents the number of identical and non-interacting sites available for ligand binding. In the presence of a potential inhibitor such as streptozotocin and at low ligand concentrations the equation takes the form:

$$\frac{[F]}{[B]} = \frac{K_d}{[B_{max}]}\left(1 + \frac{[I]}{K_i}\right)$$

Thus by plotting the free/ bound cytochalasin B against the streptozotocin concentration, the K_i can be calculated from the intercept on the x axis. Such a plot is displayed in figure 3.49. From this experiment the K_i for streptozotocin was calculated to be 1.89 mM.

A second investigation into the binding of the cytochalasin B ligand at two concentrations of streptozotocin was performed. A K_i was thus calculated from the results of an experiment where the cytochalasin B concentration was varied over a defined range. Again the results were subjected to a standard Scatchard analysis. A plot of the bound/ free against the bound cytochalasin B in the presence and the absence of 10mM Streptozotocin is shown in figure 3.50. The appropriate

Figure 3.50: A graphical plot to define the inhibition by streptozotocin upon the binding of cytochalasin B to the erythrocyte membrane. Cytochalasin B binding in the presence of 10 mM streptozotocin (black triangles) is compared to cytochalasin B binding on its own (black circles). An inhibition constant (K_i= 21.0 mM) was determined for streptozotocin.



kinetic values were determined using the equation of a straight line. A B_{max} was calculated from the intercept on the x axis, while the binding constant (K_D) was obtained from 1/ gradient.

The kinetic values calculated for the binding of cytochalasin B in the absence of streptozotocin were a B_{max} =0.80 nmol/ mg protein and a K_D= 0.18 μM. In the presence of 10mM streptozotocin the B_{max} changed to 0.78 nmol/ mg of protein, with an apparent K_D (K_{Di} =0.27 μM). The inclusion of streptozotocin with cytochalasin B resulted in an increase in the K_D for cytochalasin B without affecting B_{max}. This represents the kinetics of a competitive inhibitor, as defined by the following rate equation below:

$$B_O = \frac{B_{max} \cdot S_O}{K_D (1 + [I]/K_i) + S_O}$$

so the $$K_i = \frac{[I]}{(K_{Di}/K_D) - 1}$$

From this experiment the streptozotocin is proposed to be a competitive inhibitor with a K_i = 21 mM. An overall

inhibition constant for the binding of streptozotocin is calculated to be 11.4± 9.5 mM.

3.5.2: An investigation to determine whether streptozotocin binding is reversible or irreversible

An experiment was performed to determine whether streptozotocin binds to the erythrocyte membrane reversibly or irreversibly. Streptozotocin was initially preincubated at a fixed concentration for a range of incubation times. The streptozotocin which remained unbound to the membrane was removed by a prewash step prior to the cytochalasin B binding assay being performed. As streptozotocin was not included in the subsequent assay then any inhibition which takes place is proposed to be due entirely to that streptozotocin which has bound during the initial preincubation step. The final fixed concentration of streptozotocin which was used for this inhibition study was 10mM. This concentration represents approximately half of the observed K_i value for streptozotocin (11.4 mM).

A student t test was performed on results from two separate experimental populations. The result of the test would make it possible to establish whether the Null hypothesis could be rejected, that is irreversible binding of streptozotocin was statistically significant under the defined limits of the t test. The result of the

Student t test is stated. From statistical tables for the student t- distribution, for values exceeded in a two tailed test with a probability P. The t values for 6 degrees of freedom for both of the 5% and 10% levels of significance were 2.45 and 3.71. At the 10% level of significance there was no significant difference between any of the calculated t values and the literature values. At the 5% significance level, both of the one hour values were significantly different (t_1= 2.77 and t_2 = 3.79). The Null hypothesis is still applicable and irreversible inhibition doesn't occur. This is because calculated t values for the range of streptozotocin incubation times were not significantly different to the literature t values. Over a long time period the preincubation of streptozotocin doesn't significantly inhibit the binding of cytochalasin B. The t test supports the Null hypothesis and reversible binding as there is no justification from the t values to reject the Null hypothesis.

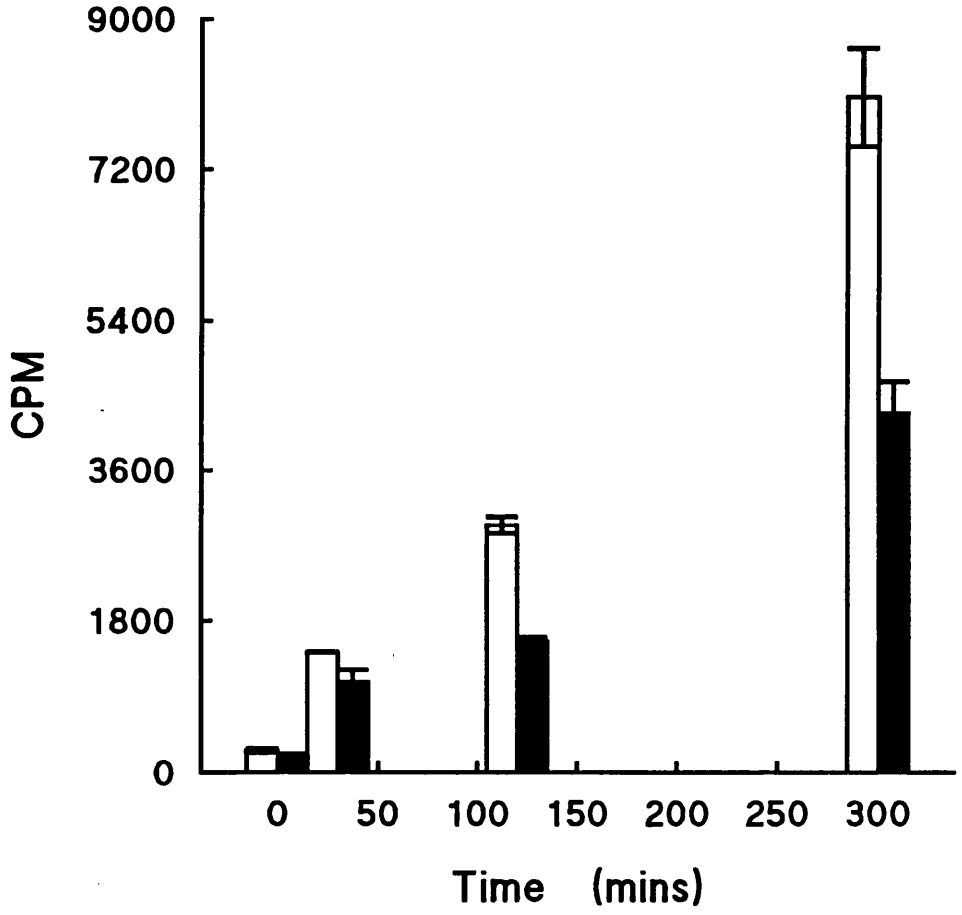
3.6: The effect of streptozotocin upon sugar transport and cell viability

3.6.1: 2-deoxy-D-glucose transport into HITm2.2 cells exposed to streptozotocin

A toxicity study with streptozotocin was performed upon the uptake of 2-deoxy-D-glucose into HIT cells. Streptozotocin at a 5 mM final concentration was included in the tissue culture medium of HIT cells, which were incubated for 48 hours. In this assay the HITm2.2 cell line was attached to the surface of the tissue culture dish. It can be inferred from the results of figure 3.51, that 5mM streptozotocin inhibited sugar uptake during 48 hours. Longer periods of incubation (greater than 48 hours) with higher concentrations of streptozotocin, resulted in the death and subsequent detachment of the cells (results not shown). This made the measurement of sugar uptake inconsistent at such time periods. Thus for the following 6-deoxy-D-glucose assays, concentrations of 20 mM STZ and time periods of 18 hours were not exceeded for obvious toxicity reasons.

An experiment was performed to obtain a K_i for the inhibition of streptozotocin upon 2-deoxy-D-glucose transport. A range of streptozotocin concentrations from 0 to 40mM were used to study any inhibition. Streptozotocin was prepared in solution prior to the

Figure 3.51: A bar graph depicting the results for an experiment performed analysing uptake of 2-deoxy-D-glucose in HITm2.2 cells. HITm2.2 cells were incubated with 5 mM streptozotocin for a time period fo 48 hours. The closed bars represent 2-deoxy-D-glucose transport in the presence of streptozotocin. The open bars represent the transport of 2-deoxy-D-glucose in the absence of streptozotocin.

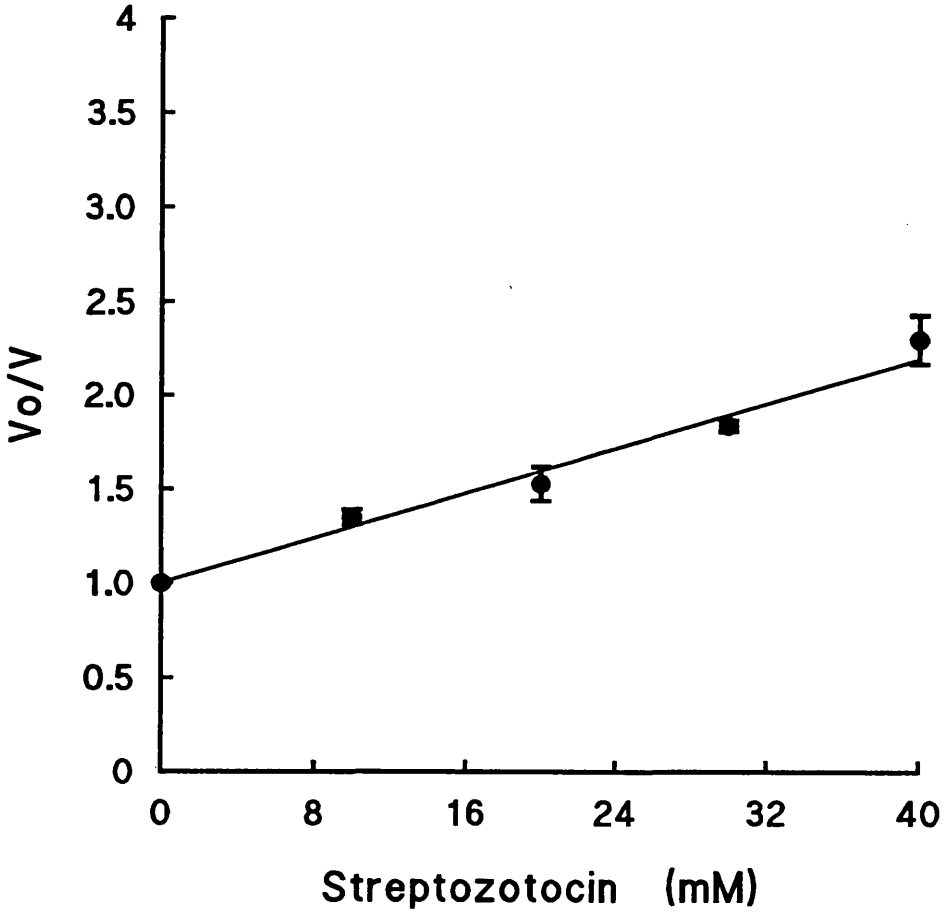


start of the experiment in order to minimise any degradation which may occur in solution. From figure 3.52 a K_i for streptozotocin was calculated in HIT cells. This value was 27.5 ± 1.5 mM for the inhibition of 2-deoxy-D-glucose.

3.6.2: 6-deoxy-D-glucose transport into HITm2.2 cells exposed to streptozotocin

All experiments here were performed with HITm2.2 cells of passage numbers 10 to 15. It has been determined that streptozotocin at certain doses targets the beta cell of the islet of Langerhans. This destruction of the beta cell renders the animal diabetic (Gunnarason et al., 1974). Streptozotocin toxicity can be prevented by the inclusion of certain D-glucose analogues, which attenuates the toxic effect (Kawada et al., 1986). The binding of cytochalasin B to a high affinity site on the erythrocyte membrane, can be prevented by certain concentrations of streptozotocin. To investigate whether the specificity of the transporter binding site can be correlated to streptozotocin toxicity, investigations with streptozotocin were performed on cell lines and combined with the data on radiolabelled STZ uptake into these lines. To determine the effect of streptozotocin, at various concentrations and time lengths upon 6-deoxy-D-glucose uptake, the

Figure 3.52: The inhibition of 100μM 2-deoxy-D-glucose in HITm2.2 cells by streptozotocin. A K_i for the inhibition by streptozotocin (27.5 ± 1.5 mM) was determined from a suitable range of streptozotocin concentrations.



following experiments were performed. All of the following graphs and tables are a representative example of two or more experiments. This is because of the variation of the 6-deoxy-D-glucose CPM'S obtained with different cell passage numbers on different days. It is difficult to account for this CPM variation between different assays on a day to day basis.

For these experiments the models of the beta cell transporter were the HITm2.2 and RINm5f cells. These were seeded and cultured in small scale tissue culture petri dishes for approximately 74 hours. During this time period both of the cell lines were exposed to various concentrations and timed incubations of streptozotocin.

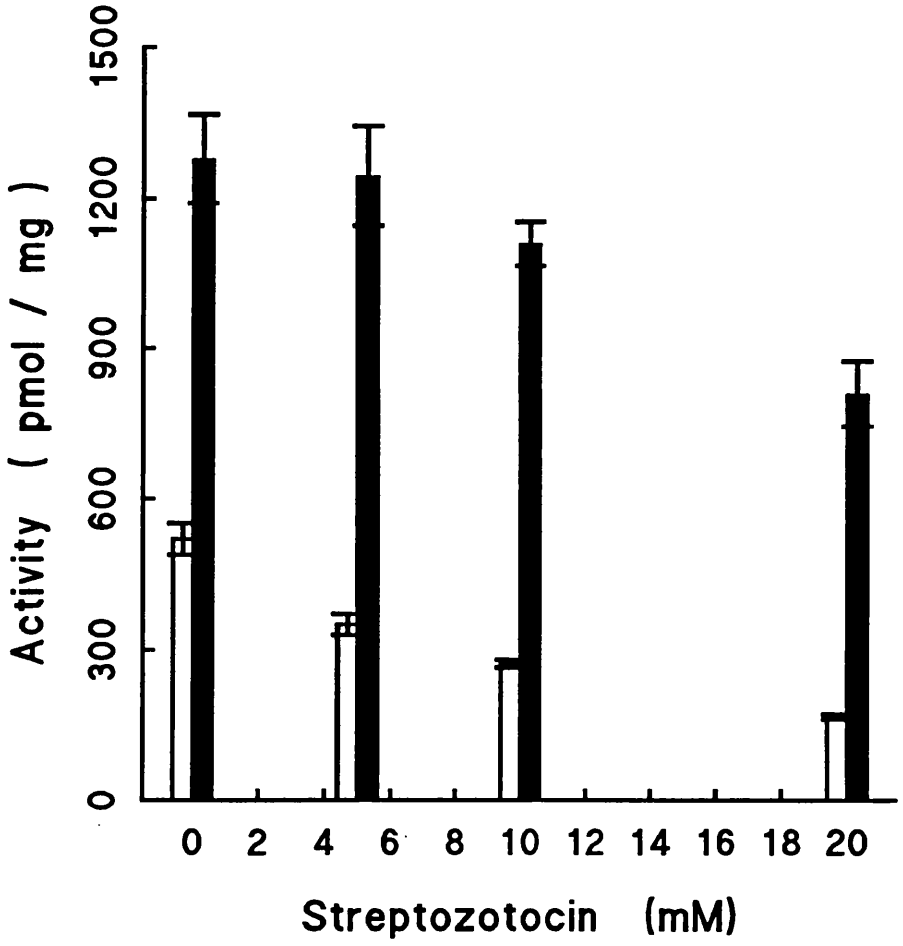
The uptake of the sugar label was performed in suspension. First the medium was removed and the cells were washed before they were detached and aliquoted in preparation for a transport assay. The relevant background zero time controls were performed, with all of the experiments being carried out in triplicate. Results were expressed as the pico moles of 6-deoxy-D-glucose transported per milligram of protein. These values were plotted against the concentration of streptozotocin or the time length of the drug exposure. In each case the magnitude of the toxicity is assumed to be proportional to the decrease in the 6-deoxy-D-glucose transported by the cell line. The activity was calculated from the following relationship:

$$\text{Activity} = (X \cdot y_s / z_t) / a$$

X is a constant of 250 pmoles of 6-deoxy-D-glucose, from a 50 µM stock concentration of the label. Y_s are the CPM'S obtained from the sample. Z_t are the total CPM'S obtained from a 5ul sample of radiolabelled 6-deoxy-D-glucose and a is the protein content expressed in milligrams. Protein concentration was calculated by using the Lowry assay (Lowry et al., 1951).

The assays with HITm2.2 cells exposed to various concentrations and time lengths of streptozotocin are illustrated in a bar chart format. From the data obtained in figure 3.53, HITm2.2 cells were preincubated for 18 hours with streptozotocin at the following four concentrations of 0, 5, 10 and 20mM. The cells were assayed in triplicate for 6-deoxy-D-glucose transport at 10 and 600 seconds. A 6-deoxy-D-glucose zero time control was performed for all the following experiments. This value was subtracted from the final results. It is observed from figure 3.53, as the streptozotocin concentration increases from 0 to 20mM there is a decrease in 6-deoxy-D-glucose uptake. This decrease is from 1300 pmol per mg at 0mM (600 seconds) to around 800 pmol per mg at 20 mM streptozotocin. Over the 10 second time period there is an initial greater activity decrease between 0 and 5mM streptozotocin. Here

Figure 3.53: A bar chart illustrating the effect of an 18 hour incubation of streptozotocin on 6-deoxy-D-glucose transport in HITm2.2 cells. Streptozotocin was incubated at a range of final concentrations (0,5,10 and 20mM). The transport of 6-deoxy-D-glucose (pmol per mg cell protein) after 10 seconds (open bars) and 600 seconds (closed bars) is plotted against the final concentration of streptozotocin. The activity in terms of sugar transport is inversely proportional to the observed toxicity.



6-deoxy-D-glucose uptake falls from 520 pmol/ mg at 0 mM to around 167 pmol/ mg at 20 mM streptozotocin.

Figure 3.54 illustrates values for HITm2.2 cells preincubated with 10 mM streptozotocin from 0.5 to 18 hours. From the results shown in figure 3.54, 6-deoxy-D-glucose transport is reduced as the streptozotocin incubation time is increased from 0.5 to 18 hours. This sugar transport reduction is most pronounced from 2 to 6 hours. Between these times there is a 100 pmol reduction in the transport activity of 6-deoxy-D-glucose. This reduction is correlated with an increased toxicity.

Figure 3.55 shows an experiment in which HIT cells were preincubated for 18 hours with 0 and 20 mM streptozotocin. The 6-deoxy-D-glucose transport activity was assayed from 0 to 5 minutes. It can be inferred from these results that there is a small reduction in 6-deoxy-D-glucose transport into HIT cells, in the presence of 20 mM streptozotocin. This reduction in transport is compared with cells incubated with 0 mM streptozotocin (control).

Table 3.3 shows data on the effects in HIT cells of selected incubation of D-glucose, 4,6-O-ethylidene-D-glucose and streptozotocin (5 mM). These three compounds were incubated for 2, 6 and 12 hours, in conditions illustrated in table 3.3. This table expresses the results of the percentage of dead

Figure 3.54: A graph illustrating the results of the effect of streptozotocin upon the transport of 6-deoxy-D-glucose in HITm2.2 cells. Streptozotocin is included in the culture medium at a final concentration of 10 mM for a range of incubation times. These incubation times are for 0.5 hours (black circles; solid line), 2 hours (black square; dashed line), 6 hours (black triangle; dashed line) and 18 hours (black star; dotted line). The 6-deoxy-D-glucose transport (activity) is expressed as the pmol transported per mg of cell protein and is measured over 0 to 300 seconds.

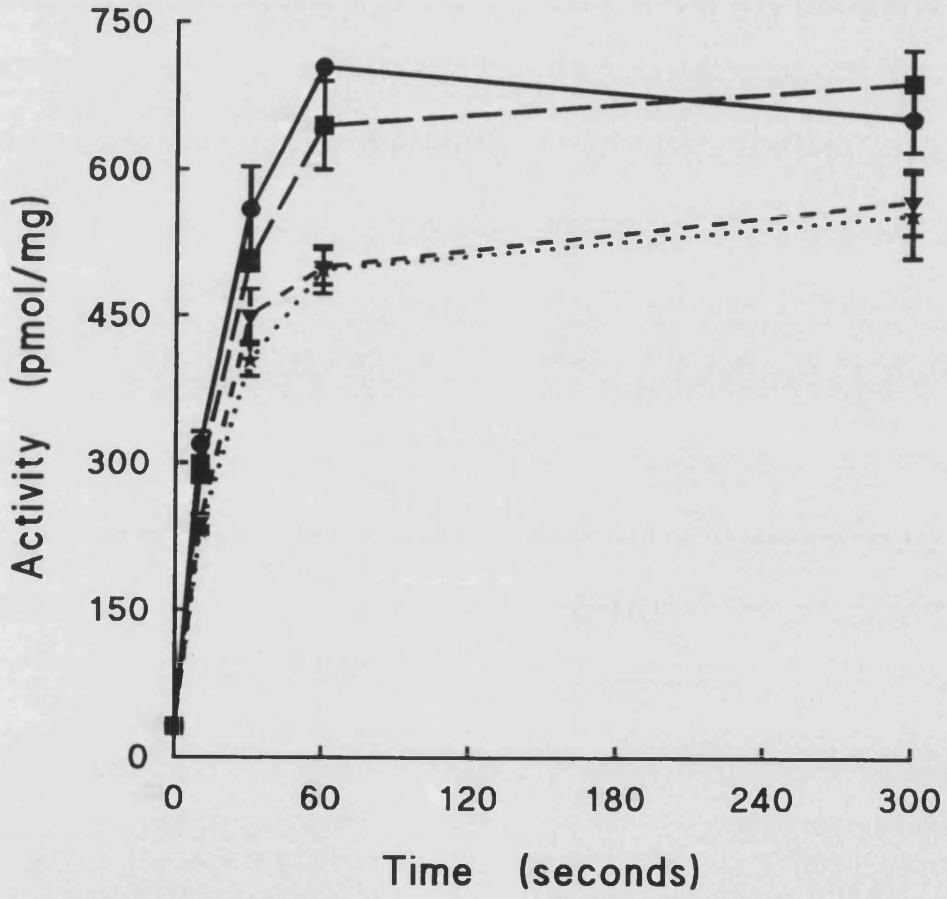


Figure 3.55: A plot showing results which define the effect of the long term incubation of streptozotocin upon 6-deoxy-D-glucose transport in HIT cells. HITm2.2 cells were incubated for 18 hours without streptozotocin (black circle; solid line) and 20mM streptozotocin (black squares; dashed line). The transport of 6-deoxy-D-glucose is measured over 0 to 300 seconds.

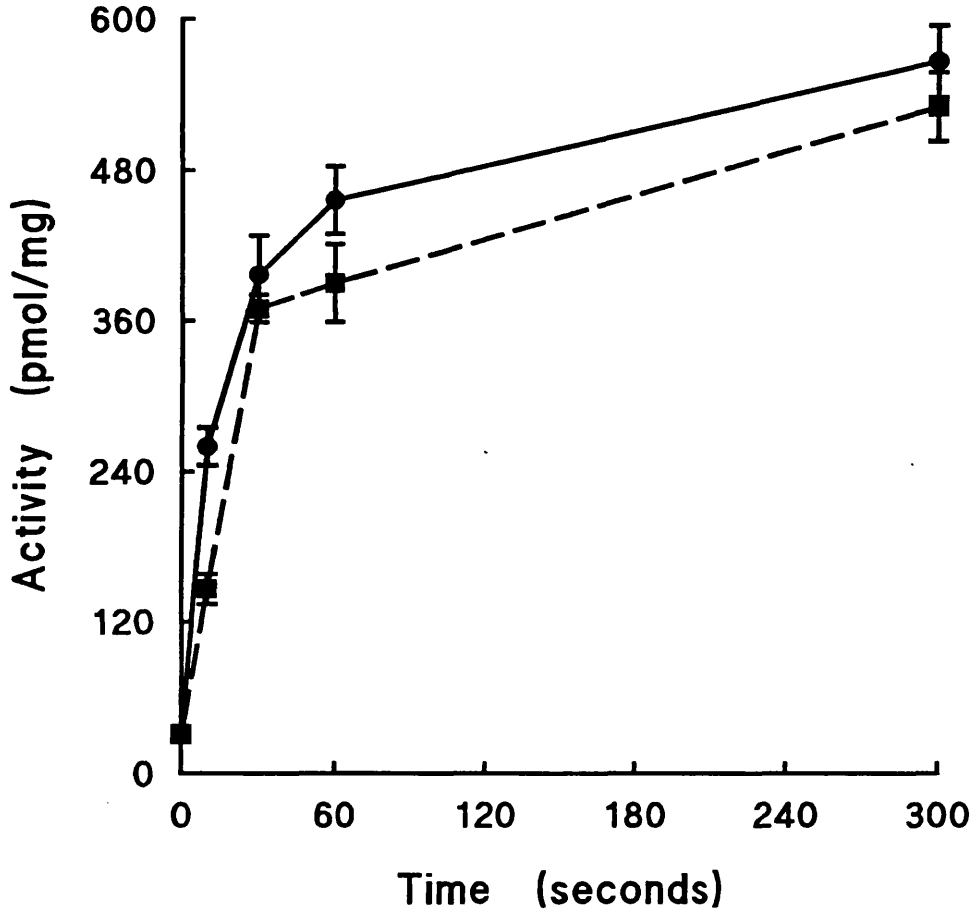


Table 3.3: A table illustrating the results obtained for HITm2.2 cells incubated with +/- glucose, +/- 4,6-O-ethylidene-D-glucose (EG) and +/- streptozotocin (STZ) for 2, 6 and 12 hours. All the compounds were added to the culture medium at a final concentration of 5 mM. The results are expressed as the percentage of the dead cells observed after the various times. The percentage of dead cells was calculated by using a standard cell staining technique. Experimental error is shown as the standard deviation calculated from triplicate values.

Incubation conditions	Percentage of dead cells (2 hours)	Percentage of dead cells (6 hours)	Percentage of dead cells (12 hours)
+ Glucose	0 %	0 %	0 %
- Glucose + STZ	34.0 ± 1.0%	63.0 ± 10.0%	97.0 ± 4.5%
+ Glucose + STZ	4.0 ± 0.1%	26.0 ± 1.4%	28.0 ± 1.4%
+ EG + STZ	1.5 ± 0.04%	18.0 ± 0.8%	void
- Glucose - STZ	10.0 ± 0.2%	30.0 ± 1.4%	65.0 ± 3.2%

cells which were calculated from Trypan Blue viability tests. In these tests normal cells were compared and used as a 100 % viable standard. HIT cells were incubated with streptozotocin in the absence of D-glucose for 2, 6 and 12 hours. After the incubation the number of dead cells were determined to be 34, 63 and 97 % dead cells for 2, 6 and 12 hours. When D-glucose was included in the HIT cell medium, the percentage of dead cells was reduced to 4, 26 and 28 %. The inclusion of 4,6-O-ethylidene-D-glucose with HIT cells resulted in 1.5, 18 and 97% dead cells being calculated for 2, 6 and 12 hours. HIT cells were also grown without D-glucose. Here the number of dead cells were 10, 30 and 65% over 2, 6 and 12 hours.

HIT cells were preincubated both with and without D-glucose and streptozotocin in the growth medium for a 2 hour time period. From the results in table 3.4, HIT cells were incubated with D-glucose and were compared with cells incubated with both D-glucose and streptozotocin. It can be inferred from these results that HIT cells grown under the two conditions, transported 6-deoxy-D-glucose at a similar magnitude for both times. This transport activity was at approximately 400 pmol/ mg and 800 pmol/ mg for 10 and 600 seconds. HIT cells were also incubated with streptozotocin without D-glucose. It could be deduced from this experiment that

Table 3.4: A table showing the effect of HITm2.2 cells grown in the presence of +/- glucose and +/- streptozotocin (STZ) at final concentrations of 5 mM. These incubations were performed for a time period of 2 hours. The 6-deoxy-D-glucose transport activity was measured over 0, 10 and 600 seconds.

Incubation Conditions	Time (seconds)	Activity (pmol per mg)
+ Glucose	0	13.0 ± 0.9
- STZ	10	345.0 ± 17.2
	600	743.2 ± 30.0
+ Glucose	10	321.1 ± 13.0
+ STZ	600	826.0 ± 5.8
- Glucose	10	254.1 ± 8.0
+ STZ	600	500.0 ± 15.0

6-deoxy-D-glucose transport was reduced to 254 pmol/ mg and 500 pmol/ mg over 10 and 600 seconds.

3.6.3: 6-deoxy-D-glucose transport into RINm5f cells exposed to streptozotocin

All these experiments were performed on RIN cells with passage numbers from 10 to 15. RINm5f cells were preincubated with 0, 5, 10 and 20 mM streptozotocin, included in the culture medium for an 18 hour time period. It was determined from results displayed in figure 3.56 that RIN cells incubated with the 0, 5 and 10mM streptozotocin, demonstrated a similar sugar transport activity. This transport was 100 to 400 pmol/ mg over 10 and 600 seconds. RIN cells were also incubated with 20mM streptozotocin. From figure 3.56, a reduction was observed in the sugar transport activity to 20 pmol/ mg over 10 seconds and 150 pmol/ mg over 600 seconds. A zero sugar uptake control was calculated and this value was subtracted from all the experimental values.

RINm5f cells were preincubated with 10 mM streptozotocin for 0.5, 2, 6 and 18 hours. In figure 3.57 the results were calculated and displayed. There was an observed decrease in sugar transport from 0.5 to 18 hours.

Figure 3.56: A graph illustrating results of the effect of streptozotocin on RINm5f cells. The streptozotocin was incubated for 18 hours at 0, 5, 10 and 20 mM final concentrations. 6-deoxy-D-glucose transport was measured over 10 seconds (open bars) and 600 seconds (closed bars), with a 0 second control being calculated and subtracted from all the values. 6-deoxy-D-glucose transport is plotted against final streptozotocin concentrations.

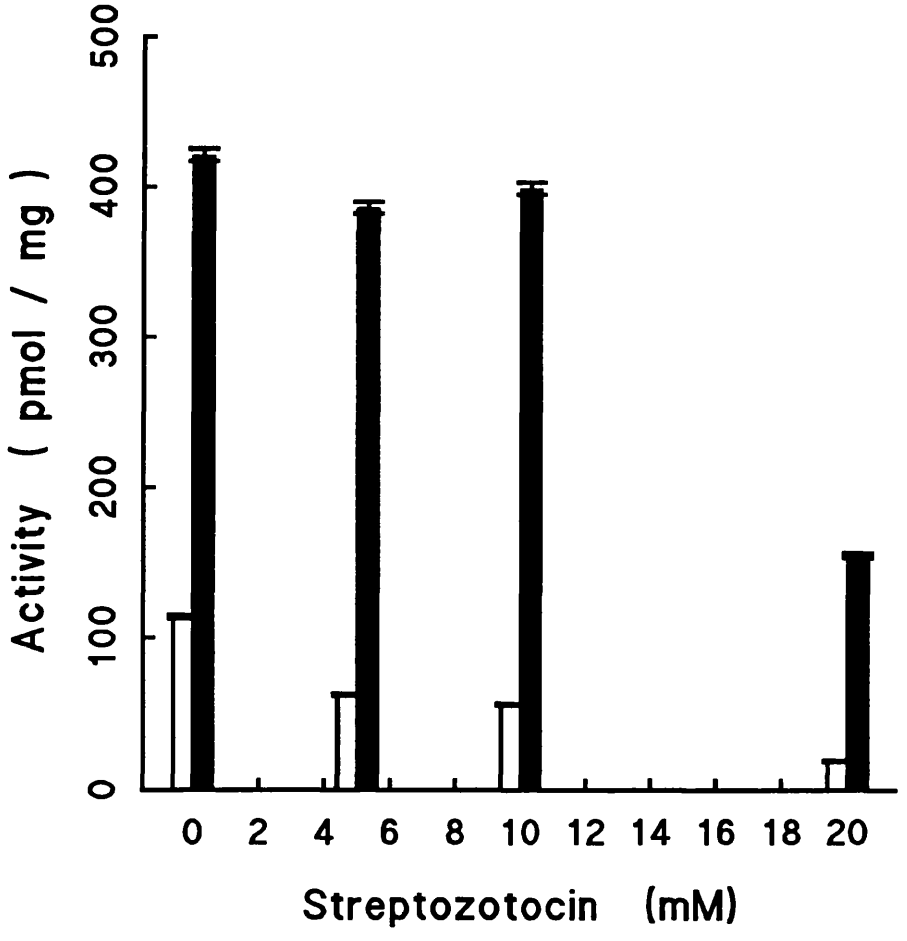
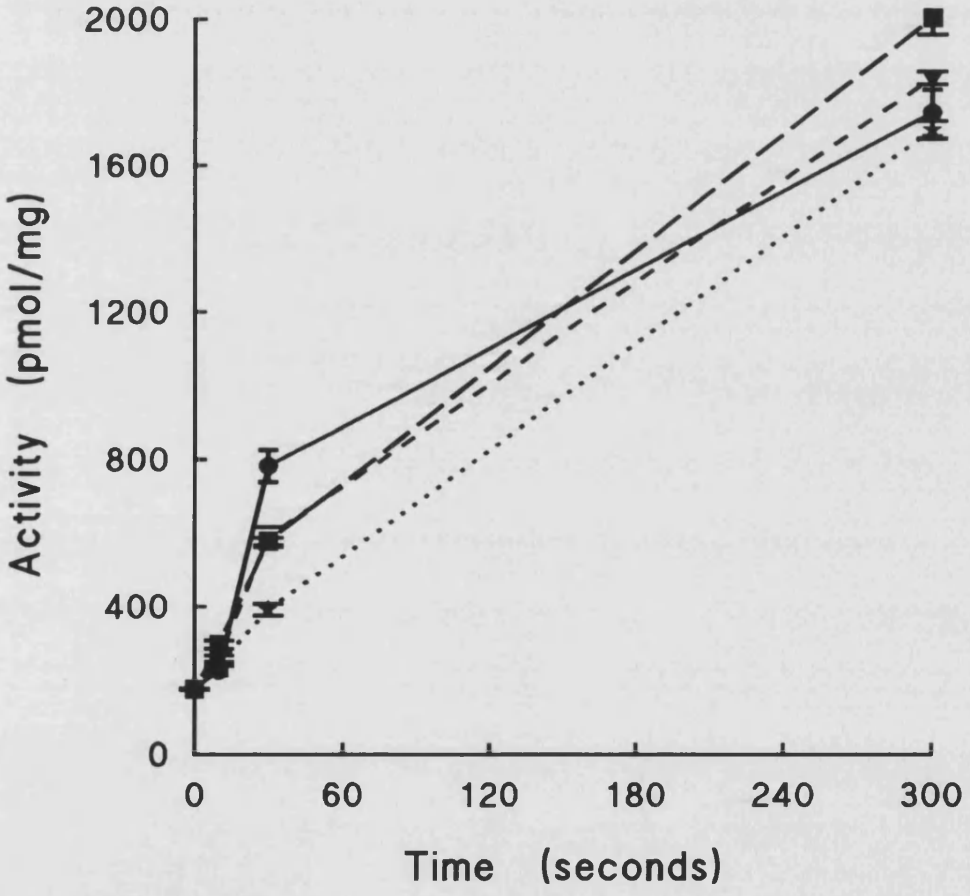
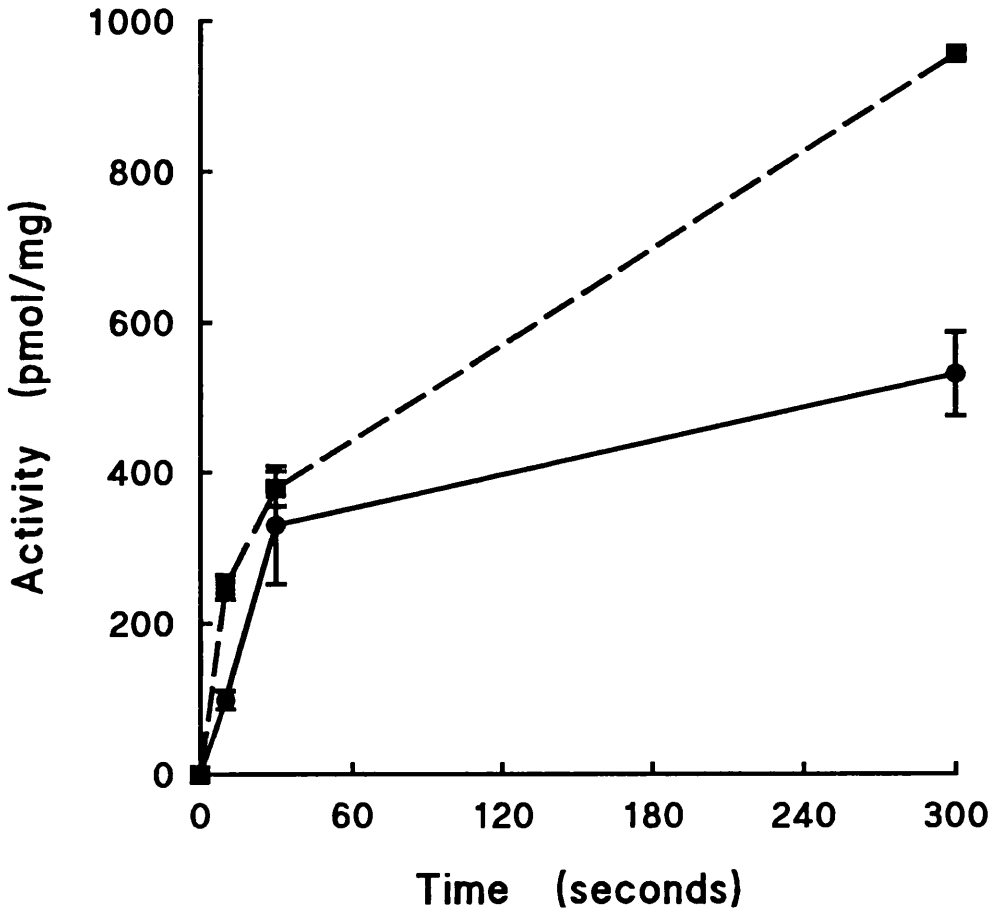


Figure 3.57: A graph illustrating experimental results of the effect of streptozotocin on 6-deoxy-D-glucose transport in RINm5f cells. Streptozotocin was included in the growth medium at a 10 mM final concentration for a range of incubation times. These incubation times were 0.5 hours (black circles; solid line), 2 hours (black squares; dashed line), 6 hours (black triangle; dashed line) and 18 hours (black stars; dotted line). 6-deoxy-D-glucose transport (pmol per mg of protein) was measured over 300 seconds.



In an experiment, RIN cells were grown for 18 hours in 15mM streptozotocin. A simultaneous control experiment was performed where cells were grown for the same time in the absence of streptozotocin. From figure 3.58, it can be concluded from these results that streptozotocin has very little effect on 6-deoxy-D-glucose transport. There is a small reduction in sugar transport in the absence of streptozotocin. This reduction in the control experiment may be due to experimental error.

Figure 3.58: A graph showing results which define the effect of long term incubation of streptozotocin upon 6-deoxy-D-glucose transport into RINm5f cells. RIN cells were incubated for 18 hours with 0mM streptozotocin (black circles; solid line) and 15 mM streptozotocin (black squares; dashed line). 6-deoxy-D-glucose transport is plotted as an activity (pmol per mg cell protein) against time (0 to 300 seconds).



CHAPTER 4: DISCUSSION

The identification of a novel islet type glucose transporter Glut 2 (Thorens et al., 1988) together with the publication of its genetic sequence, has intensified research into evaluating the precise role of this unique membrane protein. Various aspects of Glut 2 function, including its expression, genetic control and a potential site of binding for autoantibodies, are being investigated. Studies are being undertaken to determine what functional role this protein has in glucose transport and recognition in insulin secretion and in pancreatic disease states.

The binding and subsequent transport of glucose across the beta cell membrane is an important step for both the production of metabolic energy and glucose induced insulin secretion. A full kinetic characterisation of this process using a nonmetabolized glucose analogue to investigate the hydrogen bonding specificity has not previously been performed. There is a requirement to perform a thorough investigation of the glucose transport protein in islets and islet cell lines with a nonmetabolized glucose analogue. One of the aims of the work described in this thesis has been to develop a suitable transport protocol. Nonmetabolized 6-deoxy-D-glucose, has been used to study the kinetics of transport in isolation from any metabolic effects.

D-fructose was also used in this investigation as it has been reported to be specific for Glut 2 (Gould et al., 1991). Previous studies on glucose transport into free islets and beta cell lines are sparse. Many studies have used the metabolized analogue 2-deoxy-D-glucose and 3-0-methyl-D-glucose. With 2-deoxy-D-glucose kinetic studies were complicated by the phosphorylation of this sugar once it is inside the cell. Early studies (not shown in this thesis) have demonstrated that 6-deoxy-D-glucose was an effective inhibitor of 2-deoxy-D-glucose transport. Since 6-deoxy-D-glucose cannot be metabolized it was considered an important D-glucose analogue for the study of glucose transport. An additional aim, was to synthesize and study the toxicity, transport and transport inhibitory characteristics of the diabetogenic drug streptozotocin. A radiolabelled analogue was made as this was not available commercially. The binding of nonradiolabelled STZ to the glut 1 transporter was also studied. The glut 1 transporter from erythrocyte membranes is a well characterised protein. STZ binding to the erythrocyte membrane was determined by studying its inhibitory effect on the binding of radiolabelled cytochalasin B. Cytochalasin B is known to bind to erythrocytes with a high affinity (Lin and Spudich, 1974). The transport of radiolabelled streptozotocin was also demonstrated in HIT-T15 and MIN-6 cells. Here the transport of STZ and

the inhibition of this transport with 6-deoxy-D-glucose and D-fructose was evaluated. A limited amount of information was known about how streptozotocin enters cells and it was not known if STZ is transported via the glucose transporter. Evidence is presented in this thesis that streptozotocin may be transported via the glucose transporter of certain beta cell lines. Finally a toxicity study was performed in HITm2.2 and RINm5f cells. From this study the streptozotocin cytotoxicity was correlated with the available hydrogen bonding specificity information, pertaining to the hexose ring and transporter binding site. Cell viability in terms of STZ toxicity was correlated with the 6-deoxy-D-glucose transport.

4.1: Synthesis of radiolabelled streptozotocin

Initially Triphosgene was used to synthesize N-methylisocyanate (MIC). Triphosgene was reacted with radiolabelled glucosamine which resulted in the production of radiolabelled streptozotocin. Radiolabelled streptozotocin was commercially unavailable and a customised synthesis was also inappropriate. It became apparent that radiolabelled STZ had to be synthesized from glucosamine and MIC. Numerous enquiries to chemical companies regarding the purchase of MIC for the synthesis, also proved to be unsuccessful. Synthesis of

MIC had been discontinued partly due to its instability during transport and a long shipment time from the United States. It was also apparent that the production of MIC as well as being highly toxic, was also no longer commercially viable. The only alternative to this was to perform a synthesis of MIC which would enable STZ to be synthesized from glucosamine. The synthesis of MIC was performed using two protocols.

The first synthetic method was from triphosgene, which is a substitute for the phosgene reagent. Phosgene has been used for synthetic chemistry, but it is considered unfavourable due to its highly toxic side product phosgene gas. This gas is difficult to handle safely in the laboratory. Triphosgene (Aldrich, Milwaukee, Wisconsin, USA.) is a stable crystalline solid (melting point 81- 83^{o}C) which is safer and more convenient in terms of transport and storage.

A mechanism for the formation of MIC is described here. The nucleophilic methylamine will attack the carbonyl carbon of the triphosgene. For the formation of MIC to be energetically favourable, phosgene gas and the chloride anion are established leaving groups. These groups would facilitate the formation of MIC. Under the appropriate conditions the equilibrium would favour MIC formation, which in theory is then available to react with a free glucosamine molecule. Various modifications of this method were performed. A variety of solvent

systems were investigated for both the methylamine and triphosgene. The reaction was also performed in the presence of glucosamine free base. Here the hypothesis was that any MIC which was formed would react immediately with the available free glucosamine. Chapter 2 outlines in greater detail the various changes made to this method, however overall limited success was achieved. The final yield of radiolabelled streptozotocin was less than 10% and it was considered inappropriate to pursue any further investigations into the optimisation of this protocol. Upon reflection if the MIC synthesis had been scaled up and the stoichiometry of the triphosgene increased, this may have produced more of the desired end product. The limited resources in terms of time and the expense of the reagents made this impractical. This first method using triphosgene to synthesize MIC was abandoned and a suitable alternative method of production of MIC was pursued.

The second method which proved to be the more successful of the two, involved the *in situ* distillation of MIC. Here the final yield was 40% and any further protocol modifications were considered inappropriate. A better yield may have been obtained by the use of freshly prepared dichlorodiphenylsilane. This compound was shown to be unstable, as it decomposed at room temperature upon the exposure to light. Freshly distilled quinoline may have increased the MIC yield. This distillation of

quinoline had to be performed at a reduced pressure (113^{o}C at 17mm Hg) and as quinoline was purchased at the highest purity, this distillation was considered inappropriate.

Finally freshly distilled MIC was then reacted with the glucosamine free base to produce the radiolabelled end product streptozotocin. Overall the radiolabelled synthesis was successful upon following the distillation protocol.

4.2: The kinetics of 6-deoxy-D-glucose transport

4.2.1: The HITm2.2 transporter and its specificity

The transport of 6-deoxy-D-glucose in HITm2.2 cells was found to be rapid at 37^{o}C and it was necessary to reduce the temperature to 22^{o}C. HITm2.2 cells were investigated for their transporter specificity with 6-deoxy-D-glucose and D-fructose. To date there is no available literature data to make a direct comparison with these studies.

Early sugar uptake studies into HIT cells were performed with 3-O-methyl-D-glucose and 2-deoxy-D-glucose. 3-O-methyl-D-glucose was transported with a $t_{1/2}$ for equilibration of 21.7 seconds, with attainment of equilibrium within 2 minutes. This contrasted with 2-deoxy-D-glucose transport. Here the sugar never attained isotope equilibrium even after

10 minutes. 3-0-methyl-D-glucose transport data obtained for HIT cells in this thesis is comparable to similar data in islets (Lernmark et al., 1975), but contrasts to data reported in other cells lines (Meglasson et al., 1986). A $t_{1/2}$ of 18 seconds at 37 ^{o}C for equilibration of 3-0-methyl-D-glucose was reported in islets. This contrasted to sugar transport reported in other HIT cells (Meglasson et al., 1986). Here a $t_{1/2}$ for 3-0-methyl-D-glucose equilibration of 1 minute was reported and full sugar equilibration was reached in 20 minutes. The transport system expressed in HITm2.2 cells in this thesis demonstrated a high capacity for sugar uptake. This capacity was higher than that obtained in islets and other HIT cells. Transport in HIT cells in this thesis was also inhibited by the classical common sugar transport inhibitors cytochalasin B and phloretin. Therefore the transport system expressed in these cells retained recognisable transport characteristics.

The HITm2.2 cells in this thesis equilibrated 6-deoxy-D-glucose in 60 seconds (t 1/2 of 17.3 s), which was similar to 3-0-methyl-D-glucose equilibration. Meglasson (et al., 1986) reported that 3-0-methyl-D-glucose transport in HIT-T15 cells, reached equilibrium after 20 minutes. This is contrary to 6-deoxy-D-glucose transport in HITm2.2 cells. 6-deoxy-D-glucose transport rates are similar to those in dispersed islets (Lernmark et al., 1975). In these islets

3-0-methyl-D-glucose was equilibrated in 60 seconds ($t_{1/2}$= 10 seconds). Here HITm2.2 cells in this thesis demonstrated rapid transport of 3-0-methyl-D-glucose and 6-deoxy-D-glucose across the membrane. The transporter possess certain glut 2 characteristics.

The transport system in HITm2.2 cells possessed a K_i of 1.65 μM for the cytochalasin B inhibition of 6-deoxy-D-glucose transport. This can be compared to a value of 1.9 μM for the inhibition of 3-0-methyl-D-glucose in hepatocytes which contain glut 2 (Axelrod and Pilch, 1983). In islets cytochalasin B inhibited 3-0-methyl-D-glucose transport with a K_i = 1 μM (Johnson et al., 1990b).

The K_m for 6-deoxy-D-glucose transport in HITm2.2 cells was 2.07 mM with a V_{max} of 0.21 mM s^{-1}. These kinetic values were not similar to 3-0-methyl-D-glucose studies with islets. In islets the reported K_m is 17mM (Johnson et al., 1990b). V_{max} values for hepatocyte 3-0-methyl-D-glucose transport, varied from 1.3 mM s^{-1} (Craik and Elliott, 1979) to 0.005 mM s^{-1} (Goresky and Nadeau, 1974). A HITm2.2 V_{max} of 0.33 mM s^{-1} (Chen et al., 1990) for 3-0-methyl-D-glucose transport has also been determined.

Hexose transport specificity in HITm2.2 cells was studied with a range of inhibitors of 6-deoxy-D-glucose transport. The hydrogen bonding requirements were investigated with deoxy analogues of D-glucose. Spatial

constraints to binding were investigated with various hexoses, substituted with bulky groups at the appropriate positions.

Specificity at Carbon-2 (C-2)

Inhibition of 6-deoxy-D-glucose transport by D-mannose (C-2 epimer), 2-deoxy-D-glucose and D-galactose indicates that negligible hydrogen bonding occurs at C-2. Erythrocyte and adipocyte transporters (Barnett et al., 1973; Holman et al., 1981) show a similar C-2 hydrogen bonding specificity. In the intestinal sodium cotransport system (Crane, 1960) there is an absolute requirement for the gluco-configuration at C-2.

Specificity at Carbon-3 (C-3)

The K_i for 3-O-methyl-D-glucose inhibition of 6-deoxy-D-glucose transport was calculated to be 10 mM. From this kinetic value it can be concluded that 3-O-methyl-D-glucose bound with a moderate affinity to the transporter. The methyl group may contribute to some spatial interference at this position. The oxygen may partake in some hydrogen bonding with the transporter protein. This could explain the moderate affinity of this glucose analogue for the transporter binding site. 3-O-methyl-D-glucose is rapidly transported by erythrocytes, adipocyte and islet cells and is non-

metabolised (Whitesell and Gliemann, 1979). There have been some reports of the slow transport of 3-O-methyl-D-glucose by certain cultured cell lines (Meglasson et al., 1986).

Specificity at Carbon-4 (C-4)

Deoxy glucose analogues were unavailable and it can be inferred that moderate inhibition of 6-deoxy-D-glucose uptake by D-galactose (K_i= 21 mM) indicated that a glucose configuration is non-essential for binding to the HITm2.2 glucose transport protein. This transporter appears to demonstrate a fairly broad specificity. Partial inhibition by 4,6-O-ethylidene-D-glucose (Ki= 100 mM), probably due to steric interference by the ethylidene group indicates a close approach to the binding site by C-4. There is both spatial freedom and limited hydrogen bonding at the C-4 position in most mammalian transport systems.

Specificity at Carbon-6 (C-6)

The C-6 hydroxyl group is not required for any hydrogen bonding as the K_m for 6-deoxy-D-glucose indicated that the transporter has the same affinity as that for D-glucose. Hydrophobic bonding may occur at this position as 6-deoxy-D-galactose appears to demonstrate zero affinity for the transporter.

D-Fructose is transported rapidly by HITm2.2 cells with a $t_{1/2}$ for equilibration of 12 seconds. The K_m for D-fructose is 2.58 mM. From this value it can be deduced that D-fructose has a high affinity for the transporter binding site. The V_{max} for D-fructose is 0.047 mM s^{-1}, which is lower than that determined for 6-deoxy-D-glucose (Vmax= 0.31 mM s^{-1}) in HITm2.2 cells. A comparison with literature data obtained with HIT-T15 cells was made. Here a weak D-fructose competition with D-glucose uptake was reported (Ashcroft and Stubbs, 1987). D-Fructose is transported by Glut 2 but not by Glut 1 or 3. Human Glut 2 expressed in Xenopus Oocytes transported D-fructose at a rate of 0.8 pmol/ min/ oocyte (Gould et al., 1991). D-fructose also inhibited 2-deoxy-D-glucose transport in these cells.

Inhibition studies on D-fructose transport were performed in these cells. It was concluded that D-fructose only partially inhibited the uptake of 6-deoxy-D-glucose, with a K_i= 228.0 mM. Conversely 6-deoxy-D-glucose inhibited D-fructose transport (Ki = 52.0 mM) in HITm2.2 cells. From these results, it can be concluded that both D-fructose and 6-deoxy-D-glucose may bind with different affinities to the same sugar transport system in HITm2.2 cells. It would appear that the transport system in this cell line retains similar characteristics to Glut 2 and has certain differences to other reported cell lines. There may be a

separate transporter for D-fructose in these cells which also shows affinity for 6-deoxy-D-glucose.

4.2.2: The specificity of hexose transport in the RINm5f cell line

The sugar transport inhibitor phloretin at 100 μM final concentration, was observed to reduce both 3-O-methyl-D-glucose and 2-deoxy-D-glucose transport in RIN cells. From these studies the RINm5f cell line appears to posses a phloretin sensitive transporter.

6-deoxy-D-glucose is transported rapidly with an equilibrium being reached in 50 seconds ($t_{1/2}$ for equilibration= 10 s). The 3-O-methyl-D-glucose kinetics of the RINm5f transporter were previously only briefly investigated (Trautmann and Wollheim, 1987). From this work, the transporter was reported to possess a high capacity and a low affinity for D-glucose. In these cells 3-O-methyl-D-glucose was equilibrated at 37 ^{o}C within 2 minutes (Trautmann and Wollheim, 1987). In the RINm5f transporter, D-glucose (K_i =5.9 mM) has a high affinity for the transporter binding site and is able to compete strongly with 6-deoxy-D-glucose transport. D-mannose (K_i= 32 mM) and D-galactose (K_i= 52 mM) are also able to compete with 6-deoxy-D-glucose for transport. In the RINm5f cell, D-fructose was not able to inhibit 6-deoxy-D-glucose transport. This transporter appears to

be specific for D-glucose (K_i = 5.9 mM) as the latter bound with a similar affinity to 6-deoxy-D-glucose (K_m = 3.62 mM). In comparison to the RINm cells, the HIT m2.2 transporter demonstrates greater affinity for both D-glucose (Ki for 6-deoxy-D-glucose transport= 2 mM) and 6-deoxy-D-glucose (K_m = 2.07 mM). D-fructose cannot be shown to inhibit 6-deoxy-D-glucose binding in RINm5f cells. This contrasted with HIT m2.2 cells where only a small inhibition of D-fructose on 6-deoxy-D-glucose is observed. In the RIN m5f cell line, D-mannose inhibits 6-deoxy-D-glucose transport with a K_i of 32 mM, which is less than D-glucose but more than D-galactose. In the HITm2.2 cell line D-mannose bound with a stronger affinity to the transporter (Ki of 9 mM) when compared to the RIN m5f line (K_i= 32mM). D-Galactose is the weakest of the three sugars (Ki = 52 mM), while in the HIT m2.2 cells the latter bound with a higher affinity of 21 mM.

Specificity at carbon-2 (C-2)

Moderate inhibition of the carbon-2 epimer D-mannose (Ki = 32 mM), 2-deoxy-D-glucose (Ki = 23 mM) and 2-deoxy-D-galactose (Ki = 36 mM) upon 6-deoxy-D-glucose transport indicate that the 2 position may be involved in some hydrogen bonding with the transporter. It is apparent that there is limited space between the sugar and the hydroxyl group, at the C-2 position. This reduction in space is due to the interaction through

hydrogen bonding, bringing the sugar into closer contact with the protein.

Specificity at carbon-3 (C-3)

The carbon 3 position was analysed using 3-O-methyl-D-glucose. This bound to the protein with a moderate affinity (K_i = 9.5 mM). The specific binding of 3-O-methyl-D-glucose and its ability to compete with 6-deoxy-D-glucose for a mutual binding site, indicated that the 3 hydroxyl group does not participate in a hydrogen bond at this position. The tolerance of the methyl group at the 3 carbon position leads to the proposal of a degree of spatial freedom at this position. There may be space between the transporter and the sugar at this position.

Specificity at carbon-4 (C-4)

A limited analysis was performed at the Carbon 4 position. This was due to the unavailability of both the fluoro and deoxy analogues at this position. The C-4 epimer D-galactose (Ki = 52 mM), displayed a moderate inhibition of 6-deoxy-D-glucose transport and bound with a fairly low affinity. 4,6-O-ethylidene-D-glucose bound with a high affinity to the transporter (Ki = 2.36) comparable with D-glucose. It was concluded from this affinity that there was a degree of spatial freedom around the 4 and 6 positions. Without information from

the deoxy and deoxyfluoro analogues no conclusions as to the hydrogen bonding requirements could be made.

Specificity at Carbon-6 (C-6)

The effectiveness of 6-deoxy-D-glucose as a substrate (K_m= 3.62 mM) and the observation of 6-deoxy-D-galactose binding with a low affinity indicated that the 6 hydroxyl group may participate in some hydrogen bonding. D-glucose and D-galactose which contain the 6 hydroxyl group were observed to bind to the RIN transporter. As expected D-glucose bound with a high affinity (K_i = 5.9 mM), whereas D-galactose bound with only a moderate affinity (K_i = 52.0 mM). This confirms the possibility of hydrogen bonding at this position.

The RIN m5f cell line has a K_i for cytochalasin B of 10 μM. This is similar to the hepatic, islet and HIT m2.2 cells where the K_i = 2 uM. Cytochalasin B binds with a low affinity to the RINm5f transporter, which may indicate a possible structural difference at the cytochalasin B binding site. Here 6-deoxy-D-glucose has a K_m of 3.62 mM and a V_{max} of 0.43 mM s^{-1}. This is not comparable to islets where a K_m = 17mM and a v_{max}= 0.53 mM s^{-1} were reported (Johnson et al., 1990b).

D-Fructose is transported fairly rapidly ($t_{1/2}$ for equilibration of 20 seconds; v_{max}= 2.30 mM s^{-1}) and is equilibrated in around 3 minutes. The K_m for D-fructose in RIN cells is 19.5 mM. From these results, the

transporter has a moderately high affinity, high capacity transport system with respect to D-fructose (K_m= 2.58mM in HITm2.2 cells). It has been reported that hepatocyte cells may posses a separate D-fructose transporter (Okuno and Gliemann, 1986). In RINm cells, D-Fructose exhibits no detectable inhibition of 6-deoxy-D -glucose transport or vice versa. As both sugars are reported to be transported, one can infer that there may be a heterogeneous transporter population in RINm5f cells. In terms of the fast equilibration of the 6-deoxy-D-glucose sugar across the membrane, the RIN cell transporter exhibited certain Glut 2 characteristics. It is reasonable to propose that these cells may express a transporter with certain unique properties which requires further confirmation and investigation.

4.2.3: The sugar transport kinetics of the HIT-T15 cell line

Studies performed with 6-deoxy-D-glucose and D-Fructose, provided some kinetic details of the transport system present in this cell line. The 6-deoxy-D-glucose analogue was transported with a $t_{1/2}$ for equilibration of 11 seconds and it reached equilibrium in 2 minutes. The intracellular water space accessible to 6-deoxy-D-glucose was calculated to be 0.92 µl/ 10^8 cells. This value was compared to a similar value for 3-0-methyl-D-glucose

transport of 0.62 µl/ 10^8 cells (Meglasson et al., 1986). Meglasson (et al., 1986) also observed that 3-0-methyl-D-glucose was only equilibrated after 20 minutes. In the work in this thesis the transport of 6-deoxy-D-glucose compared favourably with the uptake of 3-0-methyl-D-glucose into dispersed islet cells (Lernmark et al., 1975). In islets, 3-0-methyl-D-glucose equilibration was achieved in 1 minute with a $t_{1/2}$ for equilibration of 15 seconds. In the HIT-T15 cell the K_m for 6-deoxy-D-glucose is 8.33mM. This value was compared with that obtained from islets and liver cells (Johnson et al., 1990b). In these cells the K_m was between 17 and 20 mM. HIT-T15 cells also displayed a v_{max} of 0.32 mM s^{-1} for 6-deoxy-D-glucose, which can be compared to islets where a v_{max} was obtained for 3-0-methyl-D-glucose (0.53 mM s^{-1}). These HIT-T15 kinetic values were determined at low passage numbers (10 and 15). Certain cell lines were reported to express an erythrocyte type transporter at higher cell passage numbers (above 60). One such line is the RIN cells and certain hepatocyte cell lines (Thorens et al., 1988). There are conflicting reports that other cell lines express a transporter with an islet specificity (Miyazaki et al., 1990; Purrello et al., 1991). The HIT-T15 line possessed here appears to demonstrate an islet type specificity, which is reflected in the kinetic

parameters for 6-deoxy-D-glucose and D-fructose (K_m, high capacity).

There is no literature data available to compare D-fructose transport kinetics with the data on D-fructose transport in the HIT-T15 cell line obtained here. D-fructose is transported rapidly ($t_{1/2}$= 23.2 seconds) and equilibrated in 2 minutes. The K_m for D-fructose was calculated to be 0.26 mM. It was concluded from this that the sugar has a strong affinity for the transporter binding site. The V_{max} for D-fructose was 0.37 mM s^{-1}. This value supports a transport system with a fairly high capacity for this sugar. A series of experiments looking at the inhibition of D-fructose upon the uptake of 6-deoxy-D-glucose and vice versa were performed. The results of these experiments were inconclusive and no inhibition was demonstrated by either sugar upon one another. Thus it would appear that both sugars are not transported by one and the same transporter. The liver cell is reported to possess a separate fructose transporter (Okuno and Gliemann, 1986), so the presence of a heterogeneous transporter population in this cell line cannot be ruled out. D-fructose is transported by human Glut 2 but not by Glut 1 or 3 (Gould et al., 1991).

4.2.4: The sugar transport kinetics of the MIN-6 cell line

The MIN-6 line is reported to contain a high level of Glut 2 mRNA (Miyazaki et al 1990), with low levels of Glut 1. MIN-6 cells were acquired fairly recently from Dr. Miyazaki and a preliminary kinetic characterisation was performed with 6-deoxy-D-glucose and D-fructose. This investigation provided specificity information on the type of transporter expressed in these cells. The intracellular water space in MIN-6 cells accessible to 6-deoxy-D-glucose was calculated at 0.63 µl per 10^8 cells. This can be compared to a value for 3-O-methyl-D-glucose (0.62 µl per 10^8 cells) reported by Meglasson (et al., 1986). From initial results with 6-deoxy-D-glucose studies, it was determined that MIN-6 cells possessed a high capacity transporter ($t_{1/2}$ for equilibration= 21.7 seconds), which equilibrated 6-deoxy-D-glucose in 2 minutes. Again sugar transport appears not to be rate limiting and can be compared to 3-O-methyl-D-glucose uptake into islets (Lernmark et al., 1975) and liver cells (Williams, 1968). The MIN-6 K_m for 6-deoxy-D-glucose was calculated at 4.24 mM, with a v_{max} of 0.13 mM s^{-1}. Such values could again only be compared with values for 3-O-methyl-D-glucose obtained from islets (K_m= 12 to 30 mM; v_{max} of 0.53 mM s^{-1})

(Johnson et al., 1990b). These MIN-6 cell kinetic parameters were determined using cells at a low passage number. Conclusions drawn from the kinetic data contrasted the hypothesis that certain beta cell lines expressed Glut 1 exclusively. From the preliminary kinetic data and the finding of Glut 2 mRNA being present (Miyazaki et al., 1990), the MIN-6 cell line appears to fit the model of the pancreatic beta cell line (K_m is lower than that expected for Glut 2) (Thorens et al., 1988). Here MIN-6 cells possess a high capacity high affinity type (Glut 2) islet transporter. This hypothesis was determined from results of experiments which used nonmetabolized 6-deoxy-D-glucose as the substrate.

It was also demonstrated in MIN-6 cells, that D-fructose was equilibrated ($t_{1/2}$= 35.0 seconds) in around 3 minutes. A K_m and a v_{max} for D-fructose were calculated at 7.33 mM and 0.02 mM s^{-1}. The inhibition studies performed on MIN-6 cells produced conclusive results. D-fructose was demonstrated to inhibit 6-deoxy-D-glucose transport (K_i= 60.5 mM) and 6-deoxy-D-glucose also inhibited D-fructose uptake (K_i= 23.5 mM). Thus it can be concluded that both of these sugars are transported or bind to one and the same transporter. The kinetics of this transporter are similar to Glut 2. Work for the future would be to perform a full

kinetic characterisation of the hydrogen bonding specificity in this cell line.

The synthesis of a limited quantity of radiolabelled streptozotocin enabled a brief study of its uptake. The inhibition of the transport of 6-deoxy-D-glucose and D-fructose by streptozotocin was also studied. The HIT-T15 and MIN-6 cell lines were chosen to study the anticipated transport of the radiolabelled drug. From these results streptozotocin appeared to be transported by MIN-6 and HIT-T15 cells. The latter equilibrates STZ in under 4 minutes ($t_{1/2}$= 57.8 seconds), whereas MIN-6 cells attained an equilibrium after 3 minutes ($t_{1/2}$= 31.0 seconds). Preliminary inhibition studies indicated that transport in HIT-T15 cells was inhibited by 6-deoxy-D-glucose (K_i = 28.4 mM), with no detectable inhibition being able to be shown with D-fructose. The MIN-6 cell line was different and 6-deoxy-D-glucose inhibited STZ transport (K_i = 14 mM) along with D-fructose (Ki = 33.3 mM). It would appear that there are specificity differences between the binding site in both of these cell lines, in terms of both the uptake of streptozotocin and the analogue that can inhibit this uptake. Work for the future would be to obtain a K_m for streptozotocin in these and the other cell lines. Inhibition studies could also be performed with a variety of other sugar analogues and the fungal inhibitor cytochalasin B, to determine more specificity information

on the binding of this drug and prove unequivocally that it is transported by a specific transporter.

4.3: The effect of streptozotocin upon cytochalasin B binding to erythrocytes

Prior to the availability of cell lines and as isolated islets were not easy to obtain in sufficient quantities, the affinity of streptozotocin for the cytochalasin B binding site was studied in erythrocytes.

There was some evidence to indicate that STZ was transported via the glucose carrier in both erythrocytes and islets (Kawada et al., 1987). These authors have demonstrated that 4,6-O-ethylidene-D-glucose prevented streptozotocin accumulation in rat pancreatic tissue. Transport of streptozotocin has been reported to vary in certain cell types. Pancreatic and liver cells have been shown to possess the highest recorded uptake rates of STZ (Anderson et al., 1974).

From studies in this thesis, it can be inferred that streptozotocin inhibited the binding of cytochalasin B to the erythrocyte (K_i= 1.89 mM). As there was no available literature data to compare with these studies, it was inferred that the streptozotocin bound at or near to the cytochalasin B binding site on Glut 1. From the structure of streptozotocin one can propose that the drug can interact with the glucose carrier via the

2-deoxy-D-glucose moiety. This association is believed to occur through specific hydrogen bonds with the transporter binding site. It was also predicted that streptozotocin would bind reversibly to the transport protein. This was demonstrated by a student t test which defined the long term binding of STZ, in the presence of radiolabelled cytochalasin B. The results of the test supported the Null hypothesis and that no irreversible binding of STZ to the transporter statistically occurred. If streptozotocin is transported via the glucose carrier then irreversible binding would restrict transport at the vital sites upon the protein.

From a Scatchard plot it was concluded that STZ competitively inhibited cytochalasin B binding (K_i= 21 mM). Cytochalasin B has previously been demonstrated by other authors to competitively inhibit the erythrocyte carrier with a K_i of between 0.26 and 0.4 μM (Jung and Rampal, 1977). The presence of 10mM streptozotocin caused an increase in the respective K_D (0.27 μM from 0.18 μM at 0 mM streptozotocin), without affecting the B_{max}. This is the recognisable kinetics of a competitive inhibitor and provides further evidence for specific reversible binding of streptozotocin.

From these initial results cytochalasin B (the substrate) and streptozotocin (the inhibitor) were unable to bind simultaneously to the transporter site as they were in competition for that same site or at least a

site within close proximity. It is well documented that cytochalasin B can competitively and stochiometrically be displaced by glucose and other substrate's at the glucose transporter site (Jung and Rampal, 1977). Streptozotocin can competitively displace cytochalasin B from binding to glut 1 and this is evidence that the former will bind to the glucose transporter or the cytochalasin B site close to or at the transporter. The overall K_i for the binding of streptozotocin was 11.4 ± 9.4 mM. Conformation of this was provided by the uptake of radiolabelled streptozotocin into HIT-T15 and MIN-6 beta cells which has not been previously been demonstrated.

4.4: Streptozotocin and its effect upon cell viability and the transport of D-glucose analogues into the HITm2.2 and RINm5f cells.

Experiments were performed with 2-deoxy-D-glucose and streptozotocin which was included to study its effect on 2-deoxy-D-glucose transport. HITm2.2 cells were cultured for 48 hours in the presence of 5 mM streptozotocin. It was concluded from these results that STZ produced an approximate 50 % reduction in 2-deoxy-D-glucose transport (figure 3.51). The K_i for streptozotocin was calculated to be 27.5 mM (figure 3.52). From the K_i it was inferred that streptozotocin competitively inhibited 2-deoxy-D-glucose transport. Streptozotocin appeared to

be transported by the same transporter as 2-deoxy-D-glucose, but further conformation showing the uptake of radiolabelled streptozotocin was required. Radiolabelled streptozotocin was synthesized but only a limited amount was available for a kinetic study to be performed. Streptozotocin transport was studied in HIT-T15 and MIN-6 cell lines.

From work with animal models, the destruction of the beta cell by streptozotocin can be prevented by 4,6-O-ethylidene-D-glucose and other sugar analogues being included (Kawada et al., 1986). There is limited evidence for a specific streptozotocin/ glucose recognition site being present on the beta cell membrane (Kawada et al., 1987). It is not clear whether the toxicity of streptozotocin is related to its specificity of binding to the transporter and what transport system is specific for streptozotocin transport. The sugar transporter specificity may differ between the HIT, RIN and MIN-6 cell lines and some evidence for that difference is provided here. Specificity of the sugar transporter is also compared and contrasted with glut 2.

From figure 3.53 the results calculated for HITm2.2 cells exhibited a trend towards a reduction in 6-deoxy-D-glucose transport. This reduction in sugar transport is proportional to an increase in streptozotocin concentration over an 18 hour incubation. HITm2.2 cells were incubated for times of 0.5 to 18 hours

in the presence of 10mM Streptozotocin (figure 3.54). It was concluded from this result that the exposure of cells to 50% of the final K_i concentration of STZ, appeared to decrease 6-deoxy-D-glucose transport. The greatest reduction in sugar transport was between 2 and 6 hours. HITm2.2 cells incubated for periods greater than 18 hours (results not shown) were non viable. An 18 hour incubation of HITm2.2 cells with 15 mM streptozotocin was performed. It was concluded from these results that sugar uptake was moderately reduced (figure 3.55). There are no literature experiments to compare with this HIT cell toxicity study. This toxicity data can provide evidence that streptozotocin is able to inhibit the transport of 6-deoxy-D-glucose in HIT cells. These results support the hypothesis that streptozotocin competes with D-glucose for a mutual binding site upon the membrane. This theory is also supported by radiolabelled streptozotocin uptake studies.

A viability study was performed on HITm2.2 cells which were incubated with streptozotocin, D-glucose and 4,6-O-ethylidene-D-glucose under defined conditions (table 3.3). It was concluded from the results that 4,6-O-ethylidene-D-glucose and D-glucose, added to the culture medium could prevent the cytotoxic actions of streptozotocin. From table 3.3, the number of dead cells with D-glucose and 4,6-O-ethylidene-D-glucose were reduced over 2, 6 and 12 hours, compared to the cells

incubated with streptozotocin. Over a 2 hour period there was an 8.5 fold protection from cell death due to D-glucose and a 23 fold protection as a result of 4,6-O-ethylidene-D-glucose being included with streptozotocin. A control experiment was performed on HIT cells without both D-glucose and streptozotocin. From these results it could be inferred that a portion of the dead cells was due to the absence of D-glucose in the media. The increase in HIT cells death was more pronounced at longer time periods of incubation. At 6 hours, it was inferred from this result that there was a 2 and 4 fold protection compared with only streptozotocin, by D-glucose, 4,6-O-ethylidene-D-glucose and a D-glucose absence. It can also be concluded from the results for 12 hours, that there was a 3.5, 0 and 1.4 fold protection from the death of the cells. This was due to the inclusion of D-glucose, 4,6-O-ethylidene-D-glucose and zero D-glucose. 4,6-O-ethylidene-D-glucose appeared to lose its protective capacity over a longer period of time. This suggested that it was displaced from the binding site or that cell death resulted from a lack of nourishment. A parallel glucose protection experiment against STZ was carried out over 2 hours (table 3.4). In this experiment 6-deoxy-D-glucose transport was correlated with cytotoxicity. From this study it could be observed that there was a reduction in 6-deoxy-D-glucose

transport when streptozotocin was present and D-glucose was absent.

It can be concluded from the results obtained in this thesis that the HITm2.2 cell line was sensitive to streptozotocin. There is also further evidence to propose that the D-glucose transporter was the site of uptake for the drug. Uptake of radiolabelled streptozotocin into the MIN and HIT cell lines provided evidence to support the transport of STZ via the 6-deoxy-D-glucose transporter specifically, or at a site in close proximity to the latter. Kawada (et al., 1986) have reported on the protection against streptozotocin toxicity in the beta cell which occurs with 4,6-O-ethylidene-D-glucose. This analogue has been observed to bind exofacially to human erythrocytes (Barnett et al., 1975) and rat adipocyte (Holman and Rees, 1982). From these two literature citings, 4,6-O-ethylidene-D-glucose was proposed to be an asymmetric non transported inhibitor of D-glucose transport. The induction of diabetes with STZ was thought to provide some indirect evidence for the participation of a specific transport protein in the membrane of both cultured and collagenase isolated beta cells. Other transported analogues namely 3-O-methyl-D-glucose and 2-deoxy-D-glucose (Gonda et al., 1976), are also proposed to protect against streptozotocin toxicity in islets. Researchers in this scientific field have speculated upon the existence of an unknown membrane transporter pool.

This pool of transporters may possess a unique specificity which may be similar to Glut 2. It has been proposed that such a transporter will specifically recognise the 2-deoxy-D-glucose moiety of streptozotocin.

Evidence for a specific transporter which recognised the D-glucose moiety of STZ has been reported by Kawada (et al., 1986; Kawada et al., 1987). This group performed some experiments in suitable animal models and islets, which produced evidence for the protection by 3-0-methyl-D-glucose against the toxic effect of streptozotocin. Kawada (et al., 1987) also demonstrated that 2-deoxy-D-glucose and N-acetylglucosamine exhibited protection against cytotoxicity. The alpha-0-methylstreptozotocin analogue was demonstrated to be nondiabetogenic in beta cells, with neither the counterpart sugars alpha-0-methyl-3-0-methyl-D-glucose and alpha-0-methyl-2-deoxy-D-glucose being shown to prevent cytotoxicity. The beta anomer of Streptozotocin was reported to be less diabetogenic than the alpha anomer (Rossini et al., 1977). D-mannose and D-galactose were also demonstrated to be nondiabetogenic when they replaced the 2-deoxy-D-glucose moiety on the streptozotocin molecule.

The RINm5f cell line was subjected to a similar toxicity study. From figure 3.56 RINm5f cells were incubated for 18 hours with 0, 5, 10 and 20 mM streptozotocin. From these results RIN cells are able to

tolerate streptozotocin incubation. This tolerance is indicated from the result showing a small decrease in sugar transport as the streptozotocin concentration is increased. There is a reduction in 6-deoxy-D-glucose transport in the presence of 20 mM streptozotocin. At this concentration the drug may exert other unknown nonspecific effects on the cell, not related to sugar uptake. This may indirectly effect the sugar transporter in these cells. This contrasted to the HITm2.2 cell line where the cells appeared to be more sensitive to streptozotocin. RINm5f cells when incubated from 0 to 18 hours with 10mM streptozotocin, results are demonstrated which show again that these cells tolerate streptozotocin (figure 3.57). This is correlated with a small reduction in 6-deoxy-D-glucose transport. Data for the incubation of 15mM streptozotocin for 18 hours produced results which show that these cells are able to tolerate streptozotocin. Any observed decrease in the transport activity was negligible (figure 3.58). There were no parallel literature comparisons available for RINm cells, as studies had only been performed with isolated islets. The few studies performed on RINm5f cells demonstrated that these cells exhibited a degree of tolerance to another diabetogenic agent alloxan (Sener and Malaisse, 1985). These authors have speculated that the resistance to toxicity in these cells to this agent, is involved with the D-glucose transporter binding

site. Fairly recently Ledoux and Wilson (1984) studied the effect of streptozotocin upon the RIN-r cell line. This group concluded that these cells were not as sensitive to the drug as were isolated beta cells.

4.5: A correlation of the binding site specificity with the toxicity of streptozotocin.

Table 4.1 illustrates the range of K_i s obtained for both of the HITm2.2 and RINm5f cells. The differences in specificity between these two cell lines with respect to the inhibiting sugars upon the uptake of 100 μM 6-deoxy-D-glucose were as follows. For the natural substrate D-glucose, the inhibition constants were the same within the limits of experimental error. The D-fructose sugar, while no detectable inhibition on 6-deoxy-D-glucose transport could be observed in RINm5f cells, it did appear to inhibit 6-deoxy-D-glucose transport in HITm2.2 cells with some specificity. As for the other sugars, D-mannose and D-galactose demonstrated a slightly greater affinity for the transporter binding site in HITm2.2 cells.

At the two and three positions upon the carbon ring, the hydrogen bonding between the sugar and the protein is proposed to be the same for both cell types. Each cell line was able to tolerate a bulky side group at the 3 position and this analogue bound with a moderate

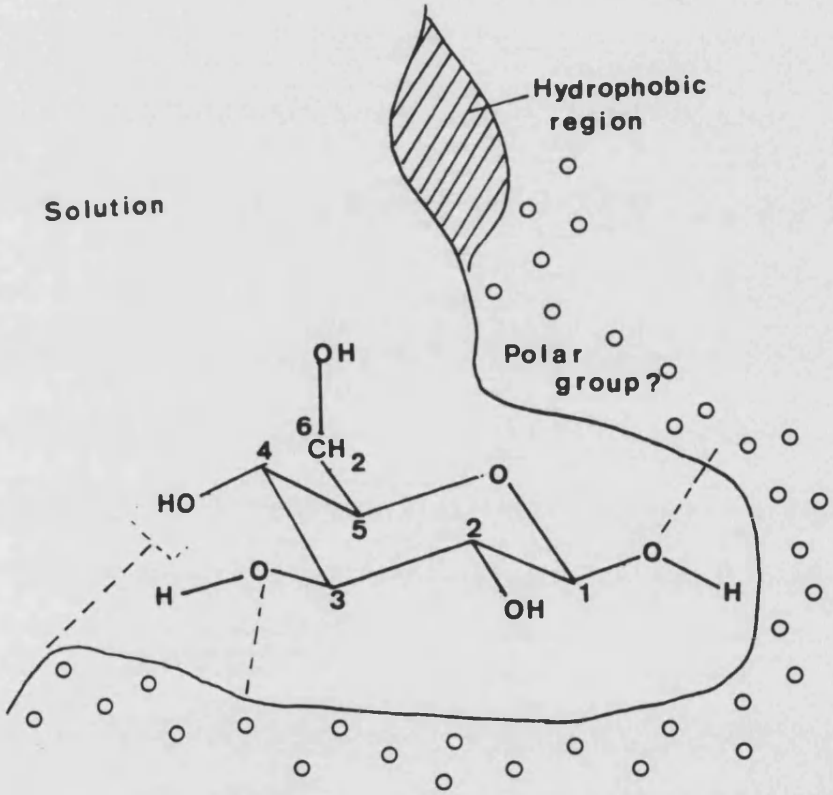
Table 4.1: A table to compare and contrast the K_i's of D-glucose analogues, on 100μM 6-deoxy-D-glucose transport in the HITm2.2 and RINm5f cells. 2DG = 2-deoxy-D-glucose, 2DGAL = 2-deoxy-D-galactose, 3MG = 3-0-methyl-D-glucose, EG = 4,6-0-ethylidene-D-glucose, 6DG = 6-deoxy-D-glucose, 6DGAL = 6-deoxy-D-galactose and N.D.I. = No Detectable Inhibition.

D-glucose Analogue Inhibitor	HITm2.2 K_i (mM) (μM where stated)	RINm5f K_i (mM) (μM where stated)
D-glucose	2.0	5.9
D-fructose	228.0	N.D.I
D-mannose	9.0	32.0
D-galactose	21.0	52.0
2 DG	6.3	23.0
2 DGAL	43.2	36.0
3 MG	10.0	9.5
EG	100.0	2.4
6 DG	2.07 (K_m)	3.62 (K_m)
6 DGAL	N.D.I.	98.0
CYT B	1.7 μM	9.4 μM

affinity to the binding site. There was a difference in affinity to the transporter observed with 4,6-O-ethylidene-D-glucose, as the latter bound with a stronger affinity to the RINm5f cell line. The 4 and 6 positions were fairly important to enable hydrogen bonding to have taken place in the HITm2.2 transporter. This was supported by the weak inhibition of the 6-deoxy-D-galactose label in HITm2.2 cells, which further supported the existence of hydrogen bonding at the C-6 position. Figure 4.1 illustrates the model for the established specificity of the Glut 1 binding site. From the specificity information obtained on the HITm2.2 transporter binding site, this cell line has important hydrogen bonding sites with the carbon 2 and 3 positions on the hexose ring. The carbon 4 and 6 positions appear to be less important hydrogen bonding sites in the HIT cell. Specificity data obtained on the RINm5f cell line give a different model for D-glucose binding to the transporter site. In this cell line, the important hydrogen bonding sites are carbons 3, 4 and 6, while carbon 2 appears to be not so important for hydrogen bonding to take place with the transporter binding site.

Finally the difference between the transporter binding site specificity in HIT and RIN cells can be correlated to the difference in cytotoxicity between these two cell lines. HIT cells exhibit 4 fold more affinity for 2-deoxy-D-glucose than RIN cells. This may

Figure 4.1: A model of the D-glucose carrier complex in the human erythrocyte (from Barnett et al., 1973). The broken lines indicate the proposed hydrogen bonding interactions between the sugar hexose ring and the transporter protein.



account for HIT cells increased sensitivity to streptozotocin as this compound has a 2-deoxy-D-glucose moiety in its structure. It is proposed that HIT cells bind streptozotocin with a greater affinity, which facilitates an increased transport of the drug. This increased binding affinity and transport will result in an increased cell toxicity in HIT cells. RIN cells only display a moderate affinity for 2-deoxy-D-glucose which may lead to a poor binding and more tolerance of the toxic effects of streptozotocin. As RIN cells were able to tolerate the toxic effects of streptozotocin at certain concentrations, it was considered inappropriate to perform any protection experiments with 4,6-O-ethylidene-D-glucose. RIN cells display up to 50 fold more affinity for 4,6-O-ethylidene-D-glucose than HIT cells. It is speculated that 4,6-O-ethylidene-D-glucose may protect against streptozotocin toxicity more than in HIT cells, due to the analogues affinity for the RIN cell transporter binding site. On the results produced in this thesis, there appears to be structural differences between the RIN and HIT D-glucose transporter binding sites. These specificity differences may account for differences in the binding and transport of streptozotocin.

4.6: Future work

A) The specificity study could be extended to clarify the requirements at the 2,3,5 and 6 carbon positions. This would establish whether the models for the substrate carrier complex proposed in this discussion are correct.

B) Further kinetic studies with radiolabelled streptozotocin are required to be performed to establish the K_m and v_{max} values for the drug. Some further work to study the specificity of the hydrogen bonding in the MIN-6 and HIT-T15 cell lines is also necessary. These studies would use the same 6-deoxy-D-glucose analogues.

C) There is a further requirement to study the toxicity of streptozotocin in these beta cell lines, correlating the cytotoxicity with the quantity of insulin secreted. An enzyme linked immunosorbant assay (ELISA) was developed for a more precise determination of the insulin secretion as a measure of the toxicity. Further studies would be performed with the MIN-6 cell line as it constitutes the best model of the beta cell. This is based on the kinetic data determined in this thesis, together with the reported insulin secretory pattern and glut 2 mRNA when compared with collagenase isolated islet beta cells. This ELISA would enable the transport of D-glucose to be correlated with the secretion of insulin in the MIN-6 cell. Cell viability could then be

established as to whether the transport of D-glucose is reduced before insulin secretion.

D) A study of the glucokinase enzyme activity together with the transport of D-glucose could also be studied with a working assay to determine the activity of the enzyme. It has been speculated that both the transporter and the glucokinase enzyme may associate to ensure the transport and identification of D-glucose, in the early part of glucose identification. It has been proposed that the function of certain intracellular amino acids upon the D-glucose transporter may take part in some form of association with the glucokinase protein. This may be essential for the initiation of the insulin secretory response to occur.

4.7: Conclusions

Glucose transport into the beta cell is the first stage in the identification of glucose and the response in terms of insulin secretion. To understand the binding specificity of the islet type transporter can only assist in the aetiology of pancreatic metabolic breakdown, namely diabetes. The work here has attempted to characterise the hexose transport process, in particular with respect to streptozotocin binding to the D-glucose transporter and the associated cell toxicity.

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